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硕 士 学 位 论 文

多氯联苯(*Aroclor 1254*)对小鼠精巢内精子发生的影响研究

EFFECT of PCBs (AROCLOR 1254) on SPERMATOGENESIS in TESTIS of MOUSE

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I am Professor **Chong-Gang Wang**, hereby certify that I have read this manuscript and recommend for acceptance by the Xiamen University a dissertation entitled "*Effect of PCBs (Aroclor 1254) on Spermatogenesis in Testis of Mouse*" in fulfillment of degree of Master of Science of Xiamen University.

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ABSTRACT（IN CHINESE）

摘要：

多氯联苯（PCBs）是一类由人工合成的氯代芳烃类化合物，被称作潜在的内分泌干扰物。由于他们具有类雌激素和抗雄激素的性质，对雄性生殖系统造成极大危害，导致不育和一些性激素依赖的生殖功能丧失。他们能够引起内源性系统改变，类似天然性激素，并抑制性激素实现其功能。

本文目的是研究多氯联苯对雄性小鼠生殖系统的睾丸生长与发育的影响。雄性小鼠随机分成五组，每组 21 只。采用不同浓度 Aroclor 1254（0, 0.5, 5, 50, 500 μg/kg）对小鼠进行灌胃染毒，每星期三次。暴露 50 天后处死小鼠，称其体重，睾丸重量和附睾重量，并进行精子计数和精子形态畸形实验。结果表示，处理组与对照组相比的小鼠体重与性腺指数并没有出现显著性差异。精子死亡率在 500 μg/kg 处理组表现出显著的上升。精子数量在 0.5 μg/kg, 5 μg/kg, 50 μg/kg 和 500 μg/kg 处理组都呈现出显著的下降。精子畸形率在 50 μg/kg 和 500 μg/kg 处理组表现出显著的上升。

用放射免疫（RIA）方法检测睾丸内性激素水平变化。结果表明，与对照组相比，PCBs 处理组的睾酮和 17β－雌二醇均没有出现显著性变化。对睾丸进行组织学观察发现，输精管的直径和支持细胞的数量与对照组相比均呈现显著性上升（$p< 0.05$），精细胞的数量呈现显著性下降，并呈剂量－效应关系，而精原细胞没有出现显著性变化。

用 Western blot 方法检测处理组与对照组之间的细胞增殖核抗原（PCNA）、雌激素受体（ER α／β）和雄激素受体（AR）蛋白水平变化。统计分析发现，处理理与对照组的 PCNA，AR, ER α 和 ER β 水平均没有出现显著性变化。在本实验中，精子质量和组织学上的变化表明环境浓度的 Aroclor 1254 能对引起雄性小鼠精子和精巢发生变化，接下来我们将对 Aroclor 1254 对精子形成的影响及其机制做进一步研究。

关键词：Aroclor 1254；PCBs；精子发生；雌激素受体；小鼠

ABSTRACT

Polychlorinated biphenyls (PCBs) are a group of halogenated aromatic hydrocarbons, synthetic chemicals which do not occur naturally in the environment. PCBs are considered potential endocrine disruptors. They are estrogen-like and anti-androgenic chemicals in the environment contain potentially hazardous effects on male reproductive axis resulting in infertility and other hormonal dependent reproductive functions. These toxic substance cause alteration of the endocrine systems, mimic natural hormones and inhibit the action of hormones.

The aim of this study is to examine the effect of Polychlorinated biphenyls (PCBs) on testicular development of male reproductive system in mice. The male mice were randomly assigned to five groups with each group comprising twenty-one members. In those mice were administered 0 μg/kg (control group) and 0.5, 5, 50, 500 μg/kg Aroclor 1254 (treated group) by gavages three time per week. Treatment was carried out for 50 days after which the mouse was sacrificed and the body weight, testicular weight; epedidymis weight, sperm mortality, sperm count and sperm abnormality were taken. However, there was no significant difference in testicular/body weight and epididymis/body weight ratio in treated group compared with the control group.

According to the analysis of sperm quality, Aroclor 1254 treated group demonstrated significant increased in sperm mortality in 500 μg/kg; decreased the sperm count in 0.5 μg/kg, 5 μg/kg, 50 μg/kg and 500 μg/kg; and significantly elevate the sperm abnormality in 50 μg/kg and 500 μg/kg compared to the control in a dose-dependent manner.

The sex hormone levels in the testes were detected by radio-immunoassay (RIA) method. The levels of testosterone and 17β-estradiol did not reveal significant alteration ($p< 0.05$) in PCBs treated groups compared to the control in a dose-dependent manner. The testis were obtained and subjected to routine histopathology following exposure to PCBs in supplement diet. The diameter of the seminiferous tubule and the number of Sertoli cells in the treated group increased significantly ($p< 0.05$) in comparison to the control group. For the spermatogenic cell, the number of germ cell in high concentration decreased significantly ($p< 0.05$). However, spermatogonia cells in PCB treated group showed non-significant difference ($p< 0.05$) compared to the control.

Western blot analysis was used to determine the level of protein between the control and treated group. The level of Proliferating cell nuclear antigen (PCNA) was determined and the results have shown no significant alteration between the treated groups and the control. the level of sex hormone receptor (ER α/β); Androgen receptor (AR) were identified in the testes to detect the proliferative effect induced by PCBs. Statistical analyses of AR, ER α and ER β did not reveal significant difference between the control and the treated groups. In the present study, we continue to investigate adverse effect of Aroclor 1254 and their mechanism on spermatogenesis. The result of Sperm quality and histopathology showed that Aroclor 1254 at low concentration induce inhibitory effect on testicular function of male mouse.

KEYWORDS: Aroclor 1254; PCBs; Spermatogenesis; Estrogen receptor; Mice.

LIST OF ABBREVIATIONS

ABP:	Androgen binding protein
AMH:	Antimullerian hormone
AR:	Androgen receptor
Ah receptor:	Hydrocarbon receptor
BSA:	Bovine Serum Albumin
EDC:	Endocrine disrupting chemical
ER:	Estrogen receptor
FSH:	Follicle stimulating hormone
GnRH:	Gonadotrophin realising hormone
HTF:	The human tubule fluid
LH:	Luteinizing hormone
NBF:	Neutral buffered formalin
OCPs:	Organochlorine pesticides
PCB:	Polychlorinated biphenyl
PCNA:	Proliferating cell nuclear antigen
POPs:	Persistent organic pollutants
PVDF:	Polyvinylidene difluoride
SER:	Endoplasmic reticulum

CHAPTER I. INTRODUCTION

I.1. PCB

Polychlorinated biphenyls (PCBs) are a group of halogenated aromatic hydrocarbons, synthetic chemicals which do not occur naturally in the environment. They are a group of biphenyl ring chemicals consisting of chlorine, carbon and hydrogen ($C_{12}H_{10-R}Cl_R$), composed of 209 congeners however only approximately 130 congeners have actually been used in chemical formulations[1] [2] [3] [4](figure 1) **.** Their production significantly increased between begun of 1929 and peaked in the 1970 because of their useful physicochemical properties and large industrial and commercial use in hydraulic fluids, heat-transfer fluids, dielectric fluids, lubricants, inks, laminating oils, paints, adhesives, dedusting agents, fire retardants, wax extenders etc. Commercial PCBs and their environmental residues contain complex mixtures of congeners and elicit a large spectrum of biological responses [2]. They are lipophilic and have very low water solubilities, and their lipophilicity increases with increasing degree of chlorination. Congeners of PCB with a lower degree of chlorination are more volatile than those with a higher degree. Pure individual PCB congeners are colorless and often crystalline. Commercial PCB mixtures are clear to light yellow oils or resins and they do not crystallize, even at low temperatures. These chemical substances are practically fire resistant because of their high flash points (170–380 °C). They form vapors which are heavier than air, but are not explosive. They have low electrical conductivity, high thermal conductivity and high resistance to thermal degradation. On the basis of these properties they have been used as dielectric isolators in electrical equipment. Similar to many organochlorine compounds, many of the congeners are highly persistent and accumulate within food chains [5]**.**

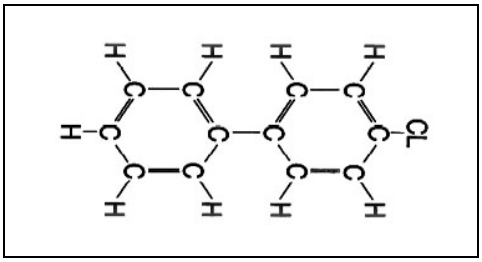

Figure 1 Chemical structure of PCBs[6].

I.2. SOURCE, ENVIRONMENTAL & HUMAN EXPOSURE OF PCBs

Various commercial PCB mixtures are known in the United States by the trade name Aroclor that have no known smell or taste [7]. The manufacture of PCBs was stopped in the U.S. in 1970 through the US EPA Toxic Substance Control Act (US Environmental Protection Agency). However, they continue to be detected as major global pollutants in the environment and cause harmful health effects [8]**.**

Recent evidences have shown that PCBs enter the environment from the soil, although volatilization that begins in the soil is not a major source of outdoor air PCB concentrations. Rather, outdoor air concentrations in urban areas appear to be on the basis of venting of indoor air. While the use of PCB was banned many years ago, they are still found in the environment today. This is because of their thermal stability, resistance to microbial degradation, and chemical inertness [8]**.** These toxic substances have a tendency to persist in the environment, with half-lives for most congeners ranging from months to years. The discharge of PCBs from the soil is slow, particularly for the more highly chlorinated congeners, and translocation to plants via soil is insignificant. Cycling of PCBs through the environment involves volatilization from land and water surfaces into the atmosphere, with subsequent removal from the atmosphere by wet or dry deposition, then revolatilization **[9].**

PCBs belong to a group of chemicals known as persistent organic pollutants (POPs); these can cause global environmental contamination. The contamination of PCB (POPs) is a significant health problem because POPs can be accumulated and magnified through the food web or food chain. They can then have several adverse effects on human health and wildlife survival. Many examples of the accidental contamination by POPs abound and the risk assessment of POPs in food is important and necessary for human health **[10].**

Occupational exposure to PCBs occurs mainly via the inhalation and dermal routes. Commercial PCB mixtures are colorless to dark brown oils, viscous liquids, or sticky resinous semisolids. Although they evaporate slowly at room temperature, the volatility of PCBs increases dramatically with even a small increase in temperature.

Overheated equipment that contains PCBs can vaporize significant quantities of these compounds, creating an inhalation hazard that can be magnified by poor ventilation. Because of their highly lipophilic nature, PCBs can also be absorbed through the skin following contact with contaminated equipment, water, or soil [9].

Among the various types of foods, fish is one of the main sources of contaminants although fish products account for only about 10% or less of the diet. POPs in fish from some areas were detected to assess the risk to human health. With the prohibition of the massive usage and production of the compounds, the residual levels in foodstuff have decreased significantly.

In China, in the late 1970s and early 1980s, considerable amount of organochlorine pesticides (OCPs) and PCBs were used. Although there is not sufficient information in China about these chemicals in edible fish from some freshwaters, however, most freshwater fish consumed come from some local lakes or reservoirs heavily polluted by POPs [10].

I.3. ENDOCRINE DISRUPTORS

Since 1990, the plausibility that some environmental and industrial chemicals could cause a number of disorders in hormonally regulated biological systems and affect human health has preoccupied biologists, toxicologists and epidemiologists. Additionally, the time of exposure during development is an indication that disturbance of the early life environment has an impact on both the child's and the adult's health[11].

Endocrine disrupting chemicals (EDCs), defined by the United States Environmental Protection Agency (USEPA), are exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior. The use of the phrase *exogenous agents* implies that in sequence for a substance to be deemed an EDC, it must come from an external source. However, an organism's environment consists of not only external factors but also an organism's internal hormonal milieu. For example, if an organism's individual endogenous hormonal systems are activated or inactivated at inappropriate times, this may disrupt endocrine processes. Another example is that of the mammalian fetus, in which the intrauterine

environment may alter endocrine and homeostatic processes. Thus, the environment needs to be redefined to include not only exogenous environmental factors, as specified by the USEPA above, but also internal secretions such as inappropriate endogenous and maternal hormones [12].

While endocrine disrupters interfere with single components of the endocrine system and indirectly can affect other hormones such as steroid hormones, they are most directly involved in the development and maintenance of reproductive function. By reason of their relatively simple chemical structure and lipophilic nature, the regulatory pathways of steroids can be easily modified by pharmacological, environmental and dietary agents. Steroid hormones bind to intracellular receptors. Upon ligand binding, steroid receptors become activated, enter the nucleus and bind to recognition sequences in promoter regions of target genes, the hormone responsive elements. Moreover, rapid, nongenomic action may be mediated by interaction with specific membrane receptors, such as those identified for estrogens and progesterone on human spermatozoa. Thus, steroid homeostasis has several critical steps including synthesis, receptor binding and transactivation, which can be the targets of different agents, including receptor agonists and antagonists as well as inhibitors of the steroid biosynthesis chain. Particular attention is given to endocrine disrupters such as the dicarboximide fungicides and the herbicide linurons which are identified as antagonists of the androgen receptor (AR) and agonists of the estrogen receptor alpha (ER-α). These cause potential damage in the development of the male reproductive tract. Now, there is an increased interest in the interactions of xenobiotics with other nuclear receptors such as ER-beta (ER-β). The nuclear receptor ER-β shows a differential distribution of splice variants in human testicular cells, suggesting specific functions in spermatogenesis[13].

The synthetic chemicals with known endocrine disrupting effects can be classified into five groups: organochlorine pesticides, dioxin compounds, polychlorinated biphenyls (PCBs), alkylpolyethoxylates (APEs) and plastic additives. In addition, phytoestrogens that occur naturally in foods have known endocrine disrupting effects. It is currently estimated that there are at least 70 chemicals in the environment which are known to be reproductive endocrine disruptors. However, of these specific chemicals in the environment, only a handful has been properly tested and those with known endocrine

disrupting properties have largely been discovered by chance **[14].**

PCBs are one of the most persistent and well-known group of endocrine disrupting compounds in the environment. These chemical compounds can interfere with normal reproductive function in humans and animals, acting as endocrine disrupters in estrogens, antiestrogens and goitrogens **[15, 16]**. Sikka et al. (2008) reported that endocrine disruptors are estrogen-like and anti-androgenic chemicals in the environment. They exhibit potentially hazardous effects on the male reproductive axis; this may result in infertility and other hormonal-dependent reproductive functions including erectile dysfunction [17, 18]. Recent evidence have shown that many endocrine disrupters can interfere with the normal hormonally regulated biological process to adversely affect the development and reproductive function in wildlife, experimental animals, and humans. These toxic substances are able to alter the normal functioning of the endocrine and reproductive systems by mimicking or inhibiting endogenous hormone actions, or modulating the synthesis of hormones **[19].**

PCBs and their metabolites act directly as agonists of estrogen. These substances can bind to plasma hormone-binding globulins, such as transthyretin (TTR), thus displacing both retinol (vitamin A) and thyroxine (T4). They induce enzymes that can change the concentrations of hormones or substrates involved in steroid hormone synthesis or signal transduction in cells. These enzymes include testosterone and hydroxylases uridine diphosphate glucuronyl transferase (UDPGT) that are important in modulating hormone concentrations. PCBs also cause neuro-behavioral deficits and developmental deformities. The mechanism by which such effects are caused is less well know, but it has been speculated that interactions with the endocrine system might be involved during early development **[20].**

Moreover, PCBs reduced fertility and ED (erectile dysfunction), testicular and prostate cancers, abnormal sexual development. Alteration in pituitary and thyroid gland functions, immune suppression, and neuro-behavioral effects are also possible due to such endocrine disruption in the male **[18]**. Recent studies demonstrated that prenatal exposure to PCBs and polychlorinated dibenzofurans can cause adverse effects on semen quality. There have also been numerous reports of adverse effects on male reproduction following prenatal or postnatal exposure to PCBs in laboratory rodents. These effects

include alterations in the weight of the testis, the seminal vesicle, and the ventral prostate, reduced serum testosterone levels, and impaired fertility. The effects of developmental exposure to PCBs on testis weight and fertility in laboratory rodents depend on the test congener or mixture, the dosage, the developmental stage during exposure, and the age of the animal at the time of examination, as well as the species and strain [16].

Krishnamoorthy et al. (2005) confirmed that these effects appear to be appropriate to PCB-induced hypothyroidism since thyroxine replacement attenuated the increase in testis weight and sperm production in Aroclor 1242-treated rats. In contrast, many authors reported that PCBs treatment significantly decreased testicular size and testosterone levels in plasma and adversely affected spermatogenic activity by disrupting epithelial organization in adult rhesus monkeys (*Macaca mulata*) [3].

In the male reproductive tract, an endocrine disruptor can affect several potential target sites. The most important of these, the testes (the male gonads), usually exist in pairs and are the site of spermatogenesis and androgen production. There are paracrine and autocrine regulations in various compartments of the testes that are under endocrine influences from the pituitary and hypothalamus. About 80% of the testicular mass consists of highly coiled seminiferous tubules within which spermatogenesis takes place. The remaining 20% consists of Leydig cells and Sertoli cells, whose main function is to establish normal spermatogenesis. The spermatozoa produced are haploid germ cells responsible for fertilization and species propagation [18].

I.4. TOXICITY OF PCBs

The absorption of PCBs occurs through the gastrointestinal tract and is distributed throughout the body. Studies of individual chlorobiphenyl congeners indicate that from 75% to greater than 90% of PCBs are readily absorbed, with oral absorption efficiency. Because of their lipophilic nature, PCBs, especially the more highly chlorinated congeners (tetra- through hexachlorobiphenyl), have a tendency to accumulate in lipid-rich tissues. Larger relative amounts of PCBs are usually found in the liver, adipose tissue, skin, and breast milk. It has been shown that absorption by nursing infants of tetra and higher chlorinated congeners from breast milk ranges from 90% to 100% of the dose. Children can also be exposed to PCBs through placental transfer. PCBs have also been

measured in other body fluids including plasma, follicular fluid, and sperm fluid. The retention of PCBs in fatty tissues is linked to the degree of chlorination and also to the position of the chlorine atoms in the biphenyl ring. In general, higher chlorinated PCBs persist for longer periods of time [21]. The major route of PCB excretion is in the urine and feces; however, of more importance is elimination in human milk. Predominately metabolites are found in urine and bile, and small amounts of parent compound are found in the feces. Biliary excretion appears to be the source of fecal excretion [22].

Disposition of PCB in Pharmacokinetic model indicates that chemical substance movement in the body occurs with exchanges between various tissues that depend on fluctuating exposure levels to specific congeners. The result is the elimination of congeners that are more easily metabolized and retention of those that resist metabolism.

The most important molecular structural factor in the determination of toxic properties and potencies in PCBs is the presence of chlorine atom on the *ortho* positions, which have coplanar structures and high affinity for the aryl hydrocarbon receptor (Ah receptor). Their toxic effects are mediated by the Ah receptor, and their toxic potencies are determined by the affinity for the Ah receptor [23].

Although there has been much research into the mechanisms of PCB toxicity, there is no clear definition of the mechanisms for most congeners. The congeners appear to act by a variety of mechanisms. Some PCB congeners are similar to dioxins and bind to a cytosolic protein, the Ah receptor, which regulates the synthesis of a variety of proteins. The toxicity of these congeners is also similar to dioxins. The toxicity of other PCB congeners seems to be unrelated to the Ah receptor. Ultimately, the toxicity of a PCB mixture may depend on the toxicity of the individual congeners and their interactions [21, 24].

The congeners PCB 153 and PCB 126 were selected as a model of PCB mixture to understand the potentially complex pharmacokinetic interactions associated with PCB mixtures. These toxics substances are the ones that appear most prevalently both in the environment and in human serum. PCB 153 is a representative nonplanar congener that appears in the environment and mammalian tissues at the highest concentration; and PCB 126 is the most toxic PCB congener with coplanar structure that binds to the Ah receptor with highest affinity. Both congeners have been suggested to induce neurobehavioral

deficits via gestational and lactational transfer [25].

These compounds produce typical AhR-mediated responses including developmental and reproductive toxicity, wasting syndrome, immunotoxicity, modulation of steroid hormone activity, and induction of multiple biochemical responses including hepatic monooxygenase activity. It has been well established in fish, birds, and mammals that the early life stages are more sensitive to the toxic effects of AhR agonists than adults [26].

Acute exposures of laboratory animals to PCBs have resulted in liver and kidney damage, neurological effects, developmental effects, endocrine effects, hematological effects, and death. Toxicity based on LD50 values for Aroclor mixtures range from about 1,000 mg/kg to more than 4,000 mg/kg. No human deaths have been associated with acute exposure to PCBs.

Numerous effects have been recognized in animal studies of chronic toxicity, including hepatic, gastrointestinal, hematological, dermal, body weight changes, endocrine, immunological, neurological, and reproductive effects. Most of the studies have involved oral exposure. Although a variety of adverse effects have been observed in animals exposed to PCBs, however, overt adverse effects in humans have been difficult to document. This has been attributed to the fact that, in most cases, the dosages tested in animals were considerably higher than those found in occupational exposures and the difficulties with interpreting epidemiological studies. These include multiple confounding factors, uncertain exposure estimates, and statistical limitations. Skin rashes and a persistent and a severe form of acne, chloracne, have been reported following exposures to PCBs. Occupational and accidental exposures have indicated that PCBs may affect many organs including the gastrointestinal, respiratory, immune, central nervous, and cardiovascular systems [21].

There is evidence that some PCB compounds are capable of disrupting reproductive and endocrine functions in human and animal [24]. Exposure of Aroclor 1254 at 25 mg/kg/day induces a significant reduction in the weight of seminal vesicles of neonatal Fisher rats and a significant reduction in spermatozoa count. Furthermore, a single intraperitoneal injection of PCB mixture has been reported to decrease testicular catalase activity in rats [27].

In adult cocks, the administration of aroclor 1254 had no significant effect on body weight and various physiological parameters. There was a marked decrease in the testicular weight and serum testosterone level. Moreover, morphological studies showed severe damage of the seminiferous tubules by these compounds [3]**.** In order to study the toxicity and biochemical effect of these compounds, avian wildlife was exposed to a complex mixture of environmental chemicals, including non-*ortho* and mono-*ortho* substituted PCBs. These congeners were found in eggs of Great Lakes birds at ng to µg PCB/g egg concentrations. They contributed a significant proportion of the total 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (TEQs) concentration to the eggs [26]**.**

The male Holtzman rats, from birth to day 9, exposed to Aroclor 1254 through early lactation exhibited decreased fertility at 18 weeks of age and increased testis weight at 23 weeks of age. The decreased fertility was not accompanied by a decrease in epididymal sperm count or changes in sperm morphology or motility, but rather a decline in the ability of sperm to fertilize eggs [16].

Previous studies report that Aroclor 1254-exposed pups manifested reduced fertility and all pups treated for Aroclor 1242 successfully impregnated females. These PCB-induced effects on sperm production and fertility also appear to be independent of changes in serum FSH or testosterone concentration, testicular histopathology, or sperm morphology or motility [28]. Recent studies investigating the reproductive effects of developmental exposure to PCBs in rodents have focused on effects in early adulthood, while few studies have addressed whether rodents at later stages of adulthood exhibit the same effects or recover to control levels. Previous studies have observed that neonatal exposure of male B6D2F1 mice to Aroclor 1254 does not adversely affect sperm fertilizing ability in vitro until 45 weeks of age [16]**.**

PCB 77 has been tested in a rat model using 100 g/kg maternal administration at day 15 of pregnancy. This dose was sufficient to cause a decrease in serum testosterone and seminal vesicle weight, whereas the testis and brain weight increased, accompanied by an increase in daily sperm production. These effects could be attributed to neonatal hypothyroidism induced by the substance during early fetal development although this has not been confirmed [14].

I.4.1. PCBs and reproductive toxicity

In the past 20 years, reproductive toxicity of PCB has been investigated in a large number of studies of humans, laboratory animals, and wildlife. Reproductive effects of PCB initially received extensive experimental examination in 1968 in connection with a reproductive failure syndrome seen in domestic mink. Many researches traced and associated the cause of this syndrome with the consumption of fish in the diet. For this reason, the database on reproductive toxicity of PCBs is unusual in that it contains extensive information on effects in mink. In humans, reproductive and developmental effects were first associated with PCB exposures in 1968 when the Yusho syndrome was identified in Japanese who consumed rice oil contaminated with commercial PCBs. Consequently, health effects associated with human exposures to PCBs via the food chain have been studied in the U.S., and in the past 20 years, PCB reproductive toxicity has been investigated in a large number of studies of humans, laboratory animals, and wildlife [4].

Reproductive toxicity is defined as adverse effects of a substance on any aspect of the reproductive cycle, including the impairment of reproductive function and the induction of adverse effects in the embryo, such as growth retardation, malformations and death. The endocrine system controls all interactions in a large extent by the body. Reproductive toxicity is categorically an advanced topic. Whereas most attention, in the past, was focused on the most dramatic aspect of birth defects, interest is now growing in the hazards for fertility [13]. Numerous studies have reported the inhibitory effects of PCBs on reproductive function, developmental abnormality, and impaired reproductive ability; the effects of PCBs in vivo and in vitro synthesis of steroids in the testis and adrenal gland or on spermatogenesis [29].

Humans produce relatively less sperm on a daily basis compared with many species of animal used for toxicity testing. The lower daily sperm production rate already places them close to the subfertile or infertile range for many men over age 30. A less dramatic decrease in sperm number, motility, and morphology in humans can have serious consequences for reproductive potential, even though it takes only one sperm to fertilize an egg. Problems in the production, maturation, and fertilizing ability of sperm are the single most common cause of male infertility [18, 30]. Although any discussion of

gonadal function and toxicity is of special relevance to man, much of this understanding has been obtained from various experimental models and research using animal species. Also, different mechanisms have been proposed to explain the presence of damaged DNA in human spermatozoa in testicular events. Among these, abnormal chromatin packaging, oxidative stress and apoptosis are the most studied. Higher levels of DNA damage means that sperm are less likely to undergo apoptosis which is a natural self-destruct process designed to rid the body of damaged cells. However, it is not clear whether increased damage arises because of chronological age or because of longer-term exposure to environmental factors that may cause such damage [18].

In the recent past, there has been increased interest in assessing the relationship between impaired male fertility and environmental factors. Fertility of men is a complex process and therefore a large variety of sites may be affected by exogenous toxic substance. PCBs cause several effects on male reproduction in vivo including impaired fertility in postnatally exposed rats, reduced matings, and decreased concentration of testicular spermatozoa, lowered weight of the testis and accessory sex organs such as epididymis, ventral prostate and seminal vesicle. In contrast, male rats exposed to Aroclor 1242 during lactation showed elevated testis weights, increased daily sperm production and increased Sertoli cell numbers per testis. Continuous exposure of lactating mothers to Aroclor 1242 causes significant changes in Leydig cell structure and function. It also causes hypotrophy and a reduced capacity to produce testosterone *in vitro* in response to LH stimulation [31].

The prevalence of testicular germ cell cancer is increasing world-wide. With the exception of spermatocytic seminomas, all germ cell cancers appear to arise from cells of carcinoma *in situ* within the testis. An increased risk of developing testicular cancer has been reported in several groups of men including gonadal dysgenic, infertile and low birth weight individuals. Briefly, all conditions that are reported to increase risk of testicular cancer are associated with a relative delay of intrauterine development or an imbalance in the activity of sex hormones during fetal development. It has been suggested that an abnormal cellular environment may enhance the survival of early germ cells which then persist in their undifferentiated form and subsequently transform into Carcinoma in situ (CIS) cells. Together with the dramatic increase in prevalence of

testicular cancer in specific geographic areas, this supports the hypothesis that environmental factors acting in utero, such as EDCs, may be responsible [14].

Moreover, declining sperm counts, increasing rates of testicular cancer and the incidence of congenital malformations of the male genital tract are increasing, largely manifested by hypospadias and cryptorchidism. On the other hand, these observations must be treated with caution since, unlike testicular cancer, the basis of diagnosis is not clear and may have been interpreted differently in various hospitals [14].

PCB mixtures and individual congeners cause hypothyroidism in rats that have been treated. This hypothyroidism can contribute for many effects typically associated with PCB exposure. Cooke (1996) reports that transient neonatal hypothyroidism in rat pups, induced by adding the reversible goitrogen 6-propyl-2-thiouracil to the mother's water from birth until Day 25, increases adult testis size and daily sperm production by 80 and 140% [32].

I.5. PHYSIOLOGY OF TESTIS

Testis has two main functions: to produce androgens (defined as male sex hormone) and male gametes [33, 34]. Spermatogenesis and steroidogenesis occur in two compartments functionally and morphologically distinguishable from each other. These compartments of testis are the seminiferous tubules and interstitium. The interstitium contains Leydig cells that produce male testosterone hormone. This hormone causes differentiation and development of the fetal reproductive tract, the neonatal organization of what will become androgen-dependent target tissues in puberty and adulthood, the masculinization of the male at puberty and the maintenance of growth and function of androgen-dependent organs in the adult. The seminiferous tubules are formed by spermatogenic cell (germ cell) and sertoli cells. In an adult, the sertoli are static nonproliferating cells that are intimately associated with and support germinal cells involved in spermatogenesis (the production of spermatozoa). The germinal epithelium is populated by cells that produce to spermatozoa [35]. The integrity of these compartments is necessary during the production of quantitative and qualitative sperm. The function of testis and its compartments are primarily influenced by structures of the hypothalamus and the pituitary gland (endocrine regulation). In addition, local control mechanisms

(paracrine and autocrine factors) play an important role in the regulation of testicular function [33].

I.6. CYCLE OF SEMINIFEROUS EPITHELIUM

The seminiferous epithelium exhibits a regular morphology which is composed of two populations of cells, the somatic Sertoli cells and the spermatogenic cells. The germ cells are in constant proliferation and maturation for the formation of new spermatozoa[36]. In mammals the seminiferous epithelium demonstrates that spermatogenic cells advance from the most basal layers in the direction of the tubular lumen, since a sequence of transformations are processed to form the spermatozoa. The germinative cell passes through different phases that are organized in a series of well-defined cellular associations or stages. These stages succeed in an area of the seminiferous epithelium and the cyclical emergence of these stages constitutes the process called as the seminiferous epithelium cycle. To quantify the process of spermatogenesis, some aspects are essential like the knowledge of the seminiferous epithelium cycle, the characterization of the stages composing this cycle, and the determination of its frequency, since these basic aspects associated to testis biometry supply the spermatic production rate [37].

The cycle of the seminifeorus epithelium has been studied in various mammals [38]. It has been defined as a series of changes in a given area of the seminiferous tubule between two appearances of the same developmental stage or cell association. Fourteen stages in the rat cycle based on the 19 phases of spermiogenesis identified by the periodic acid Schiff (PAS) stain have been detected. In effect, if it was possible to observe the same region of the seminiferous epithelium by phase contrast microscopy over time, the appearance would progress through the 14 stages before stage I reappeared. Thus, it was also demonstrated that the duration of any one stage was proportional to the frequency with which it was observed in the testis. As type A spermatogonia in any one area of the epithelium progress through meiosis and spermiogenesis to become spermatozoa. In each progression, the progeny of the spermatogonia progressively move toward the lumen of the tubule [39].

In spite of the broad uniformity of testicular function among mammals, both the spermatogenic process and the interstitial tissue show distinct interspecific differences

and it has been recognized that the amount of sperm produced is directly associated with testes size. However, the amount of sperm production also depends on the rate and efficiency of the spermatogenic process. Hence, differences in the total spermatogenic cycle duration, as well as in the length and composition of spermatogenesis stages, may also affect the amount of sperm produced. Furthermore, the location and size of lymphatic vessels, number of Leydig cells, and abundance of intertubular connective tissue also differ greatly among species [40].

A most recent study showed that the various generations of germ cells in the testes of mammals are not associated at random with a group of Sertoli cells, but form cellular associations and each association is constant. Many studies have approved these cellular associations or stages of the seminiferous epithelium into an order and correctly predicted that the 4-5 generations of germ cells which are associated with a Sertoli cell sequentially develop through the stages. The period required to pass through all stages is considered to be one cycle of the seminiferous epithelium, and the number of cycles which occurs from an initial division of a stem spermatogonium to the release of its daughter spermatozoa into the lumen of the seminiferous tubule is determined by the number of generations of germ cells associated with a Sertoli cell. The value of recognizing the stages of the seminiferous epithelium and arranging them into an order is that it provides a basis for studying how spermatogenesis is regulated and for estimating the duration of spermatogenesis and the daily rate of sperm production of an animal [41].

I.6.1. Spermatogenesis

Spermatogenesis is a highly regulated process of cell differentiation that is essential for sexual reproduction [42]. Spermatogenesis occurs in the tubules where morphologic alternations lead to the formation of differentiated sperm [43]. In seminiferous epithelium are located Sertoli cells (somatic cells); and several types of germ cells (spermatogonia A-type, and B-type), developing germ cells (primary and secondary spermatocytes), spermatides and spermatozoa (figure) [44]. Clermont (1972) define sperrnatogenesis as the process by which a spermatogonial stem cell gives rise to a spermatozoon; this can be divided into three distinct phases. The first phase concerns the spermatogonia, which proliferate to produce spermatocytes and simultaneously maintain their number by renewal. The second phase involves the primary and secondary

spermatocytes, which go through the process of the meiotic divisions leading to the formation of haploid cells, the spermatids. The third phase concerns the spermatids, which go through a complex series of cytological transformations leading to the production of the spermatozoon. The spermatocytes throughout meiosis have similar morphological characters in the various mammalian species [45].

The spermatogonia, the primitive germ cells of the male, are located at the basement of the seminiferous epithelium. Two types of A spermatogonia can be distinguished, originally from a physiological and cytological morphology. These include (a) the dark spermatogonia, which do not show any proliferating activity under normal circunstances and should be considered the stem cells of spermatogenesis and (b) the pale spermatogonia. The pale (Ap) spermatogonia, in turn, divides and differentiates into two B spermatogonia. From the B spermatogonia are derived directly the preleptotene spermatocytes before the beginning of the meiotic division. The latter germ cells commence DNA synthesis [33].

Germ cells developing from a committed spermatogonium are connected by intercellular bridges. This provides cytoplasmic continuity among members of a group of cells, except where cell death eliminates a connecting cell [44].

Type A and B spermatogonia were also identified in man. Since the identification of the types of spermatogonia largely depends on the morphological characteristics of the nucleus, the fixation that precipitates the chromatin to various degrees becomes an important factor in a study of these cells [45].

The spermatocytes are the cells that undergo meiosis, i.e., the two successive divisions leading to the production of the haploid cells, the spermatids. Primary spermatocytes are produced by the mitotic division of Type B spermatogonia and lose their contact with the basement membrane of seminiferous tubule. After their formation, the nuclei of primary spermatocytes show a remarkable similarity to the nucleus of the type B spermatogonia and the nuclear diameter being slightly smaller in the former than in the latter. Successive replication in configuration of chromatin starts with the appearance of filamentous thread like chromosomes called leptotene. Their large nucleus and extensive cytoplasm containing either coarse clumps or threads of chromatin recognize primary spermatocytes that can be seen in chromosomal division. In rat and

other rodents the nuclei of the interphase primary spermatocytes demonstrate coarse flakes of chromatin associated to the nuclear membrane, while in men and monkeys the chromophilic chromatin tends to be more granulo filamentus [45].

The secondary spermatocytes are originated from the first meiotic division. These germ cells contain a haploid chromosomal set in duplicate form[33]. Their interphasic nuclei are smaller in size than primary spermatocytes, contain in addition to a pale stained granular chromatin several globules of chromophilic chromatin free in the nucleoplasm or associated with the nuclear envelope. These cells enter in second maturation division, resulting in formation of the haploid spermatid [45].

The **spermatids** are spermatogenic cells that were produced by meiosis; undergo an extraordinarily complex series of changes, leading to the production of the highly differentiated germ cell recognizable as spermatozoon [45, 46]. In mice, spermiogenesis can be classified into three phases of spermatids; round spermatids, elongating spermatids, and elongated spermatids [43]**.** Formations of new spermatids are characterized by a small spherical nucleus and the usual array of cytoplasmic organelles, the Golgi zone, mitochondria, and centrioles. There is in addition a peculiar chromophilic mass floating in the cytoplasm called the chromatoid body [45]. During the process of spermatogenesis, the nucleus of the spermatides becomes smaller, condensed and less granular until they assume pointed form of spermatozoa. The daughter cell of each division remains connected to one another by narrow cytoplasmic bridges which only break down upon release of spermatozoa into the lumen of the seminiferous tubules [47]**.**

Spermatogenesis in humans is different from the process in bulls, mice, rabbits, rats, stallions, or other common mammals. Obvious differences include the 3-dimensional (3D) organization of the seminiferous epithelium and low number of sperm produced daily per gram of testis. The pattern of spermatogonia renewal and proliferation is also different. The difference in sperm production per gram of testis parenchyma is important to clinicians and clinical epidemiologists. Variation among human testes is much greater than that among individual mice, rabbits, or rats used by most experimentalists [44].

I.6.2. Sertoli cell

Sertoli cells can be defined as a somatic constituent of the testis which plays an essential role in spermatogenesis. Sertoli cells play a critical function in the formation of the testis; extending from the base to the apex of the seminiferous epithelium, and are in direct physical association with all types of germ cells [48]**.** In mammals and other species, two types of sertoli cell can be identified at the electron microscope level. Type A Sertoli cells which have fine structural characteristics and a light staining nucleus, contain numerous filaments and ribosomes, abundant smooth endoplasmic reticulum (SER), and typical junctions with adjacent Sertoli cells and germ cells. Type B Sertoli cells are less numerous and are found only in close opposition to the basement membrane of the seminiferous epithelium. They are small cells with dark nuclei and limited cytoplasm containing SER and numerous filaments. They are difficult to identify as they are similar in appearance to adjacent differentiating spermatogonia, at the light microscopic level **[49]**.

Sertoli cells support spermatogenesis during maturity and embryonic development. Mature ones provide germ cells with structural and nutritional support, assist their movement, produce seminiferous fluid and support spermiation. Individual SC can support only a finite number of germ cells, therefore the ultimate adult testis size and eventual sperm production is directly linked to the total SC number. Evidence from depleted Sertoli cells in rats indicates that the number of adults' spermatids depends on the number of Sertoli cells produced during perinatal development **[48].** These cells are also sensitive to a number of toxicants; they secrete Lactate and Androgen binding protein (ABP) which are products of reflect the normal function of the cells; and free radicals such as hydroxyl radical, superoxide anion, hydrogen peroxide, attack lipids, sugars, proteins and DNA **[50].**

Sertoli cells control and co-ordinate all the processes relating to testicular development and masculinisation. These cells secrete antimullerian hormone (AMH) that is responsible for regression of female reproductive ducts in the male foetus. They can regulate the regulation and multiplication of early germ cells and also the differentiation and function of Leydig cells, which produce testosterone, hormone responsible for the masculinisation of the whole foetus.

In the foetal and neonatal period, the increase of Sertoli cells is crucial as the number of Sertoli cells determines the final adult testis size and the capacity for sperm production. Studies in animals have clearly shown that when the window of Sertoli cells proliferation has been changed, there is a consequential increase or decrease in their numbers.

Although the mechanism of regulation and multiplication of Sertoli cells multiplication is not known, yet some hormones are thought to be involved in these processes. These include FSH, which stimulates the proliferation of Sertoli cells; and the thyroid hormones T3 and T4, which seems to be involved in determining the cessation of Sertoli cells proliferation. Many experiments in rats have shown that hypothyroidism prolongs the time of Sertoli cells proliferation and results in a higher number of Sertoli cells, bigger testis and higher sperm output in adult life. In contrast, hyperthyroidism causes premature cessation in Sertoli cells proliferation and finally results in lower testis size and lower sperm production[51]. The determination of Sertoli cell efficiency is another very important parameter to evaluate testis function, which is the best indicator of spermatogenic efficiency (daily sperm production per gram of testis [DSP/G/T]). This approach is based on the information that each Sertoli cell is able to support a limited number of germ cells in a species- specific manner, and also because of the fact that the number of Sertoli cells per testis is established before puberty in mammals [52].

I.7. LEYDIG CELL

In rat, Leydig cells appear initially in the testis during day 15 of embryonic development. These fetal Leydig cells secrete high concentrations of androgens that are required for Wolffian duct development and subsequent male sexual development[53]. Leydig cells are the principal source of androgens in the male. The most prominent ultrastructural features exhibited by these cells are an abundant smooth SER and fairly numerous mitochondria. Lipid droplets are common in Leydig cells of some species, although not in those of adult laboratory rats. The biosynthesis of testosterone is catalyzed by enzymes located predominantly on membranes of the SER and in adjacent cytoplasm, although a few steps occur on the inner mitochondrial membranes [23].

Leydig cells store cholesterol in lipid droplets in their cytoplasm and use the cholesterol to form androgens. These cells produce Estrogens which are able to inhibit spermatogenesis. Administration of estrogens suppresses spermatogenesis in adult male rats and can result from estrogen-mediated suppression of LH. This mechanism decreases secretion of testosterone by Leydig cells; otherwise it may indicate an intratesticular role for estradiol. It is possible that several factors involved in intraovarian signalling and involved in intratesticular signalling, such as IGF-1, may regulate spermatogenesis. In the young rat, Sertoli cells can also convert androgens to estrogens [54].

The Leydig cells were clustered around small centrally located blood vessels while the lymphatic sinusoids occupied the periphery of the interstitial areas. The endocrine cells were thus found to be interposed between the blood vascular system and the lymph [55]. Morphologically, the Leydig cells have round or polygonal shape; and core eosinophilic cytoplasm with lipid droplets of that are used as a source of cholesterol for production of male hormones, testosterone and dihydrotestosterone (DHT) [56].

I.8. HORMONAL CONTROL and FUNCTION of TESTIS

Aragon et al. reports that the regulation of spermatogenesis in mammalian organisms includes chemical communication between the hypothalamus–hypophysis axis and the gonad itself [57]. The critical importance to reproductive function is the integration of the hypothalamus, pituitary and testis. The hypothalamus comprises the lateral walls of the lower part of the third ventricle of the brain. The pituitary, an endocrine gland connected to the hypothalamus at the base of the brain, is divided into two major parts: the neurohypophysis and the adenohypophysis. The neurohypophysis (posterior lobe), which is composed of the median eminence, infundibular stem and infundibular process, receives neural input from the brain. In contrast, the adenohypophysis (or anterior lobe), composed of the pars tuberalis, pars intermedia and pars distalis, is a glandular tissue and, must be regulated by factors delivered via the circulation [58].

The testicular function requires stimulation of normal luteinizing hormone (LH) and follicle stimulating hormone (FSH) which are produced and secreted by gonadotrophin realising hormone (GnRH) in hypothalamus. LH and FSH control steroidogenesis and gametogenesis in testis (figure 2) [33].

The secretions of FSH are regulated by the negative feedback effects of the steroid hormones produced by the testis. Testosterone negatively feeds back at the level of the hypothalamus slowing the GnRH pulse generator and thereby inhibiting pituitary LH pulses. Moreover, the testis is capable of metabolizing testosterone to estradiol via aromatase activity in the seminiferous tubules and interstitium. Estradiol, when present in physiological concentrations, is also able to dampen the frequency and amplitude of episodic LH release. The effect of this negative feedback is apparent in men following castration; the loss of testicular steroids results in markedly increased secretion of both LH and FSH. When these men are given exogenous testosterone, LH levels in the blood diminish, and LH pulsatility returns to normal. The importance of the integration of the hypothalamic-pituitary-testicular axis is obvious in light of the critical roles of LH, FSH, and testosterone in spermatogenesis. Testosterone, regulated by LH, is an absolute requirement for normal spermatogenesis. FSH plays a significant role in the initiation of spermatogenesis at puberty but its role in the adult is less certain [58].

The androgens are principal regulators of male sexual system. In the adult, they are required for the maintenance and function of the male genital organs and spermatogenesis. In the human body, there are two main androgens: the testosterone and dihydrotestosterone, which is the 5 **α**-reduced derivative of testosterone. Each of them has its hole and target tissue [33]

The Testosterone is secreted by the adult Leydig cell under LH stimulation; it acts via androgen receptors [59] on Sertoli, Leydig, and peritubular cells. That testosterone exerts its effects on somatic cells instead of germ cells were highlighted by recent germ cell transplantation studies in which spermatogonia from AR-deficient animals developed into spermatozoa in wild-type recipients. FSH acts via specific G protein-coupled surface receptors located exclusively on Sertoli cells. FSH has a most important function in the development of the immature testis, particularly by controlling Sertoli cell proliferation [57, 60].

There is evidence that Estrogen Receptors (ERs) are involved in the physiology of male genital system and their action is mediated by estrogen receptor proteins, which are expressed in the male reproductive tract. ERs occur in two forms, the classical ER alfa subtype and the novel ER beta subtype subsequently discovered in rat, human, and mouse.

Both subtypes have been detected in testes and epididymis of different mammals such as the rat, mouse, human, dog and cat, but their cellular expression often varied across the species [61]. In humans, the nuclear estrogen receptor is encoded by two separate genes, the ERβ gene that has been mapped to band q22-24 of chromosome 14 and the ERα gene which has been mapped to the long arm of the chromosome 6. ERβ is homologous to the earlier identified mammalian ERα, particularly in the DNA-binding domain (95% amino acid identity) whereas the ligand-binding domain shows 55% of amino acid identity therefore, they can differ for the ligand-binding affinity. Furthermore, the ligand-binding cavity of ERβ is significantly smaller than the ligand-binding cavity of ERα, which may have implications for the selective affinity and pharmacology of ligands [62].

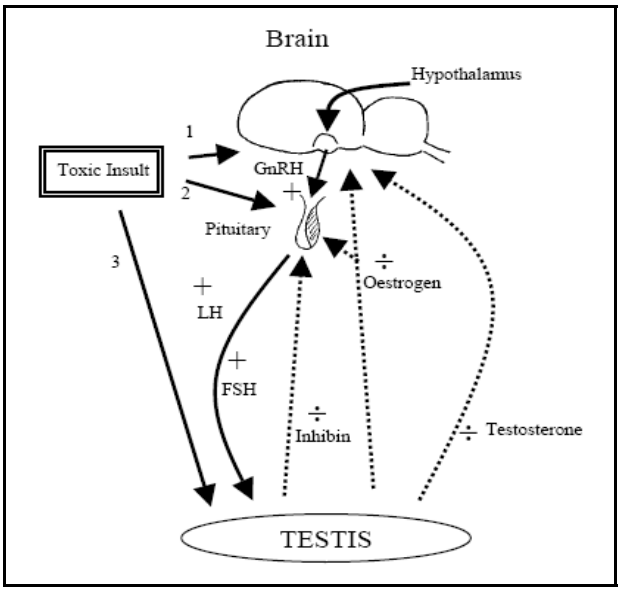

Figure 2. The neuroendocrine control of gonodal function presents a number of target sites.

The toxic substances may act directly on the (1) brain, the hypothalamus, (2) pituitary and (3) testis, therefore impairing hormonal communication among these structures [63].

AIMS OF STUDY

In the present study, it was intended to investigate the effects of PCBs on morphological change of spermatogenesis in mice's reproductive system; evaluate the morphological study of the body weight, testicular weight, epidydimis weight, sperm

count, sperm morphology and sperm abnormality; determine the concentration of 17β-estradiol and testosterone by radioimmunoassay (RIA) to investigate the effect of PCB on testis development and mechanisms; evaluate the histological and morphological consequences of PCBs exposure on seminiferous tubules diameter, spermatogenic cell and sertoli cell; investigate the organization of the spermatogenic cell including spermatogonia, spermatocytes, and spermatides in the seminiferous epithelium; determine the level of protein PCNA; and identify the level of sex hormone receptor (ER alfa, beta and AR) in the testes to detect the proliferative effect induced by PCBs using western blot analyses with β-actin as control.

Objectives

The objectives of this study were to:

- Examine the effect of PCB on reproductive system of mouse:
- Evaluated the PCB effect in body, epididymis, testis weight; and in sperm count, sperm morphology and sperm abnormality.
- Determine histological effect of PCB on spermatogenic cell and sertoli cell in seminiferous epithelium
- Analyze the level of protein and sex hormone receptor in testis of mouse

SIGNIFICANCE OF STUDY

Many researchers have demonstrated that PCBs cause numerous adverse health effects in animals and human. Some of studies have been inconclusive and they have failed to show a link between PCB exposure and diseases, basically because epidemiologic studies have limitations affecting their ability to find health effects. Those limitations are the small numbers of persons often involved in studies, difficulty in determining actual exposure levels, and multiple confounding factors as smoking, drinking, and exposure to other contaminants. Other studies in humans suggest that PCBs have both carcinogenic and noncarcinogenic health effects. Consequently, the U.S. Environmental Protection Agency (U.S. EPA) classifies PCBs as probable human

carcinogens of medium carcinogenic hazard. Exposures of PCBs in animals cause serious and long-lasting reproductive effects, such as longer menstruation and decreases in fertility, conception, and sperm rates. Moreover, PCBs can reach a fetus through the placenta and can be transferred to a newborn via breast milk. Studies of the effects PCBs have on the male reproductive system remain inconclusive. One study of men occupationally exposed to PCBs indicated no fertility problems, while another showed an association between low sperm count and high levels of PCBs in the blood[64] [51].

Therefore, the present study was conducted to discover the adverse effect of PCB at low concentration on spermatogenesis in mammals and to gain insight into the mechanism of such effect.

CHAPTER II. MATERIAL AND METHOD

II.1. CHEMICALS and EQUIPMENT

II.1.1. Chemical

Araclor 1254, a commercial PCB mixture was purchased from Sigma-Aldrich (sigma – Aldrich, Co, USA) and its purity cannot be accurately determined. The human tubule fluid (HTF) medium and antibodies to mice androgen receptor (AR) and proliferating cell nuclear antigen (PCNA） were from Chemicon International (Temecula, CA). Antibodies to estrogen receptor (ER-α and ER-β) were obtained from Bioss Co. (Beijing, China), 17β-estradiol and testosterone Radioimmunoassay (RIA) kits for determining sex hormone were purchased from Furui Biological Engineering Co. (Beijing, China). All other chemicals were of analytical grade and were obtained from commercial sources. PCB was dissolved in 100% ethanol and diluted with 0.85％ sodium chloride to obtain the final PCB concentrations were 0.1, 1, 10 and 100 μg/ml respectively and the final ethanol concentration was 1ml/10ml volume.

II.1.2. Experimental equipment

The equipment used in this experiment were 202-1-type organization of hand slicer, Sartorius electronic balance, Beckman J2-MC high-speed refrigerated centrifuge, at room temperature centrifuge, DYY-11124D small double vertical electrophoresis tank, Olympus B× 41-type microscope, GNP-9160-type water-resisting constant temperature incubator. Microplate Reader Bio a Tek's SnyegyrHT, Germany Eppendoff Kayo guns, medical gavage needle, Kadok gel image analysis system, Quantity one protein analysis software, PH meter, MAXIMIX vortex mixer.

II.2. ANIMALS and TREATMENT

All animal experiments were conducted according to the research protocols approved by Xiamen University Institutional Animal Care and Use Committee. Male C57 mice, aged 21 days and weighing 12–14 g, were purchased from Fujian Medical University, China, housed at 24±1 ˚C under a 12:12 h light–dark cycle, with free access to food and

tap water. After quarantine period, 105 mice with adequate weight gain and without clinical signs were divided randomly into five experimental groups (21 mice per group). Thus, there were no differences of statistical significance among groups in body weight and these pubertal mice were orally administered by gavages once every 3 days with PCBs (0.5, 5, 50 and 500 μg/kg, respectively). Control mice received an equal volume of vehicle (5 mL/kg). Body weight was recorded on the day of orally administered necropsy and actual dosing volumes were adjusted according to the body weight recorded.

II.3. COLLECTION OF TISSUE SAMPLE

Before dissecting, each mouse was weighed on a balance to measure the total body weight. After 50 days of treatment, the mice were sacrificed, autopsied, and their testis weighed.

Organ weight measurement

The body weight; right testis and left epididymis were measured. The left epididymis was used for sperm count, viability and deformity.

Counting sperm

The epididymis was carefully separated from the testis, and a small aliquot of sperm suspension is diluted with 1ml of HTF and minced with scissors, incubated in 5% CO_2 in air for ten minute at 37˚C. The homogenization-resistant sperms were counted using a haemocytometer (Neubauer's counting chamber). An aliquot from the suspension was taken in leukocyte hemocytometer and diluted with phosphate buffered saline. The suspension was well-mixed and charged into Neubauer's counting chamber. The total sperm count in 8 squares (except the central erythrocyte area) of $1mm^2$ each was determined and multiplied by 5×10^4 to express the number of spermatozoa/epididymis [65].

Sperm abnormality evaluation

For evaluation of sperm abnormality, twenty microliters of aliquot of the mixture was smeared to microscope slide and fixed with methanol. After fixation, the samples was stained with 1% Eosin-Y solution for 1 h, washed with distilled water, passed through neutral resin, and mounted with a coverslip. Two hundred and fifty sperms from each sample were evaluated microscopically and classified as follows: normal, bent at

cephalocaudal junction defect, detached head, acephali, small head defect, amorphous head defect, bent tail defect, coiled tail defect. Also, percentages of abnormal sperms were calculated. The proportion of abnormal sperm to total number of sperm counted within the objective field is used to calculate the percent of abnormal sperm [66].

II.4. HISTOLOGICAL EXAMINATION

The whole testis of mice were dissected; fixed in neutral buffered formalin (NBF) for 24 hours; washed in running tap water for at least 12h; dehydrated (removed water) in different concentrations of alcohol; clearing with xylene until the tissue became clear and transparent at room temperature; embedded in paraffin to dehydrated transparent sample; then the paraffin embedded testis were sectioned at 4-6 μM. the sectioned tissue were stained with Hematoxylin – Eosin (H&E) for histological examination (Morphometric Techniques).

II.4.1. Morphometric Techniques

By means of morphometrical techniques, quantitative information on the variations was observed in seminiferous tubule diameter. For each slide (control and PCB treated group), 10 seminiferous tubule cross sections were counted per animal (3 animals in each group) and tubule diameters were measured using the NIS-elements 3.0 software. Random fields from each animal were selected for differential cell counts of seminiferous tubules. For each mouse, 10 round or nearly round seminiferous tubules were selected randomly and numbers of germinal cells were measured for each tubule. Tubules were selected at 40X magnification while stages were not considered and then measurements were done at 100X magnification. Sertoli cells, spermatogonia, primary spermatocytes and spermatides were counted in each tubule according to the shape of their nuclei and cytoplasm.

II.5. WESTERN BLOT ANALYSIS

II.5.1. Sample pre-treatment

Reagente:

For the preparation of sodium phosphate buffer (pH 7.4) were used:

• 50 mM potassium phosphate (0.5 M K_2HPO_4) - 71.7 ml

• 150 mM NaCl (0.5 M KH_2PO_4) – 28.3 ml

• NaCl (pH 7.2) – 8.8g

• ddH2O - up to 1L

Homogenization of testis:

The testis of each mouse (1gram, triplicate) was homogenized in 300µL of buffer in homogenizator buffer. The sample was centrifuged at 12,000 rpm for 20 min at 4 ˚C and the supernatant was collected for further analysis.

II.5.2. Protein quantification

In this procedure were used an improved commassie brilliant blue G-250 (CBB) for colorimetric determination of protein concentration. Protein concentration was determined according to Bradford using Bovine Serum Albumin (BSA) as standard curve. The protein was quantified from the supernatant by spectrophotometer, and the level of protein PCNA and AR, ER-α and ER-β in each group was determined respectively.

II.5.3. Western blot procedure

The Western blot procedure was followed for the electrophoretic transfer of proteins on to the PVDF membrane. The gels for the Western blot were first run on electrophoretic system (described below). After transferring the protein on the gel to the membrane, the gel was stained with coomassie brilliant blue G-250 (after 1h or overnight) to identify the region of interest. The staining was performed until the background was clear and the protein bands distinct.

II.5.3.1. Poly Acrylamide Gel Electrophoresis and western blot reagent formulations

Reagents:

• Acrylamide-bis Acrylamide (30%)

• 3M Tris-HCL pH 8.8 – Resolving Gel Buffer

• 0.5M Tris-HCL pH 6.8 – Stacking Gel Buffer.

• 5xElectrophoresis Buffer: 15g tirs-base and 72g of glycine dissolved in 700 mL double

distilled water, then add 5g 0.5%SDS solution, fixed volume to 1000 mL.

• 5×SDS-PAGE Loading Buffer: 250mM Tris-Cl (pH6.8), 0.5%(M/V)BPB, 10%(M/V) SDS, 50%(V/V)Glycerol, 5%(M/V)2-ME/0.25M DTT

•10×Transfer Buffer: 190 mmol/L glycine, 25 mmol/L Tris-base. Weigh 14.4 g of glycine, 3 g Tris base, add water to total 1 L.

• Transfer Buffer: 10×Transfer Buffer 100mL+ ddH2O 900 mL+100 mL methanol.

• TBS (1L): Tris-base 2.4g, NaCl 8g, KCL 0.2g, ddH2O 800mL, HCL pH adjusted to 7.6 with 4N HCl.

• Blocking solution: To prepare the blocking solution, 0.16 mg gelatin per 40 ml Tris buffer Saline Tween20 (TBS-T) buffer (mix well in microwave) was weighed and then added to 0.4 mg BSA

• Ab Dilution Buffer: Take the amount of bovine serum albumin (BSA), diluted with PBS to 2%

• Preparation of SDS-PAGE gel: see table 2.

• TEMED (Tetramethylethylenediamine)

• Ammonium persulfate (APS)

• 0.5M Tris-HCL pH 6.8 – Stacking Gel Buffer.

• SDS

Table 1- preparation of 12% and 10% Gel

Reagents	**Resolving Gel (12%)**	**Resolving Gel (10%)**	**Stacking Gel**
DdH20 (ml)	1.28	2.93	0.68
Acrylamide-bis Acrylamide (30%)	1.6	1.67	0.17
Stacking Gel Buffer			0.13
Resolving Gel Buffer	1.04	1.3	
Ammonium persulfate – APS (µl)	50	50	10
TEMED (µl)	2	50	2
SDS (µl)	40	3	10

II.5.3.2. Performance of Gel Electrophoresis and western blot

Preparation of loading sample:

For each sample made, the dilution was thus: 0.8µl sample + 19 µl ddH2O + 5µL 5×Loading Buffer. As described in Colombo et al (2003) method [67], the sample aliquots were heated in boiling water for 5 minutes. From this, 20 µL was loaded onto the 8% or 12% gel for the performance of discontinuous SDS PAGE. The electrophoresis was performed at 80V for the sample to enter and pass through the stacking gel and at 120V from the time the samples entered the resolving gel to the end of the run. This was indicated when the bromophenol blue dye reached the end of the gel. Later, the gels were removed and they underwent the western blot procedure.

Electrophorectic transfer of proteins on to PVDF membrane

The blotting pads were immersed in transfer buffer and pressed down to remove trapped air bubbles. The soaked pads were left in buffer until the blotting sandwich were ready to be assembled. The polyvinylidene difluoride (PVDF) membrane was pre-wet in pure methanol and placed directly in to transfer buffer to equilibrate. Then a gel sandwich was made with the Gel, PVDF membrane, and paper. Scotchbrite pads were taken carefully to prevent trapping of air bubble by wetting all materials with transfer buffer before the assembly of the sandwich. This assembly was then placed in the apparatus tank and the tank, filled slowly with ice cold transfer buffer. The transfer was done at 100V during 1 hour. These procedures were conducted at 4 degrees during electrotransfer procedures.

Immunodetection

Following the SDS-PAGE, the proteins were transferred to PVDF membranes and incubated overnight at room temperature with blocking membrane gelatin. After incubation overnight five monoclonal antibodies specific for different target proteins the testis of mouse were used. These antibodies were β-actin (control), PCNA, AR, ER-α and ER-β. The membrane was incubated with primary antibody solution at room temperature for 2 hours/10 rpm, washed with TBS-T, 3 times/15 min. then with second antibody, goat anti-rabbit immunoglobulin G or goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase, the membrane was incubated at room temperature for 1h: 30 min. After incubation it was washed with wash buffer, 3 time/15 min.

Enhanced chemiluminescente detection

After washing, the blot was transferred to a shallow tray and developed following the Pierce Chemiluminescence HRP substrate instruction. The ECL reagent was added on the blot, and then it was exposed to X-ray film for the appropriate time period. For best results, it was exposed for one minute and ten seconds to visualize the chemiluminescence signal corresponding to the specific antibody-antigen reaction. The intensity of bands was quantified using Quantity One software (Bio-Rad).

II.6. HORMONES LEVELS MEASUREMENT

II.6.1. Sample pre-treatment

Individual testis was cut in to small pieces of 0.1 g wet weight for analyzing. Each samples were homogenized in ethanol (5 mL) and frozen at −80 ◦C for at least 24 h. Homogenates were extracted at first with diethyl ether (8 mL) and in two further extractions with 4:1 diethyl ether:ethanol (2× 10 ml). After, the sample was dried by liquid nitrogen and then dissolved in 2 mL 80% methanol, washed with 5 mL petroleum ether to the solution two times to remove lipids. This experiment follows same procedure described in Bettin et al. (1996)[68].

Determination

The levels of 17β-estradiol and testosterone were measured by using Radioimmunoassay (RIA) kits from Beijing Furui Biological Engineering Company. There was no cross-reactivity for antibody estradiol, estriol, testosterone, progesterone, and analytical detection limit were 5-500 pg/mL. However, the cross-reactivity was less than 0.1% for testosterone antibody, estradiol, estriol, cholesterol and detection range was 0.5 - 10 ng/mL.

Determination of radioimmunoassay kit

Briefly, the hormone concentration, different standard of sample (0.5 mL), ^{125}I-T/I^{125I}-E2, anti-serum markers were mixed and incubate at 37 ℃ for 1h. After incubation,

mixed at room temperature, put it aside for 15 min, centrifuged at 3500 rpm for 20 min. The supernatant were discarded and the precipitated radioactivity was measured in tube counts (cpm). From these data a standard binding curve was drawn as semi-logarithmic standard curve and the concentration of the sample (hormone) were determined from the curve.

II.7. STATISTICAL ANALYSIS

Statistical analysis was done by using SPSS program. Results were reported as mean±S.E. The data were statistically analyzed with one-way analysis of variance and the significance level between data was examined by least significant difference (Dunnett; $\alpha = 0.05$) post-hoc tests.

CHAPTER III. RESULTS

III.1. TESTIS WEIGHT, EPIDIDYMIS WEIGHT, BODY WEIGHT AND SPERM PARAMETERS

Body weight

There were no evident signs of clinical toxicity or gross findings in the male mice from any of the treatment groups. Data for body weight are presented in Table 3, and the final body weights of the mice were not significantly affected by the administrations of Aroclor 1254 compared to the control.

Testis weight and Ratio Testis/Body Weight:

Exposure to Aroclor 1254 in male mice did not reveal any alteration in testis weight and Ratio Testis/Body Weight. The result indicated that there were no significant difference between control and treated group in dose dependent manner (figure 3 and table 3).

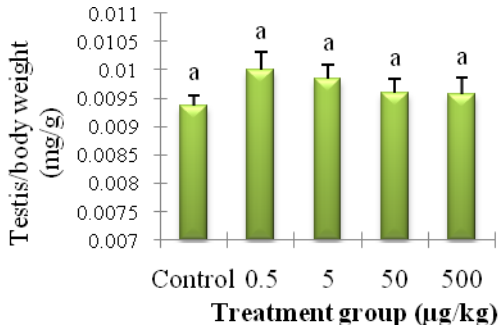

Figure 3. Relation of testis/body weight of control mouse and PCBs treated group.

The values are given as mean±SE and the result did not reveal significant difference. Treatments not sharing a common letter are significantly different ($P < 0.05$).

Epididymis and Ratio epididymis/body weight

As table 3 showed, Aroclor 1254 did not cause change in Epididymis and Ratio epididymis/body weight. There was no significant difference between control and treated group in dose dependent manner (figure 4 and table3).

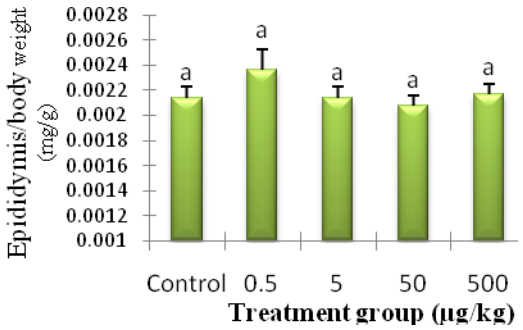

Figure 4. Relation of epididymis body weight of control and PCBs treated group.

The values are given as mean±SE and did not reveal significant difference. Treatments not sharing a common letter are significantly different ($P < 0.05$)

Sperm mortality

The Sperm mortality was increased by Aroclor 1254 treatment in a dose dependent manner, and the result showed (figure 5 and table 3) significant increase ($P<0.05$) in sperm mortality treated group (500 µg/kg) compared to the control group.

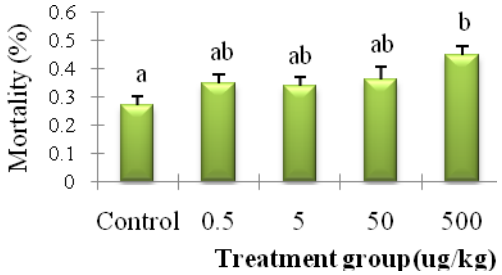

Figure 5. Sperm mortality of mice treated with PCBs.

Data is presented as Mean±SEM. There were significant alteration (P□ 0.05), between high concentration (500 µg/kg) and control group. Treatments not sharing a common letter are significantly different ($P < 0.05$).

Sperm count

Table 3 and figure 6 showed that high dose exposure (50 and 500 µg/kg) of Aroclor 1254 in male mice originated more subtle reduction of sperm count than detected at low doses (0.5 and 5µg/kg). The result demonstrated that there was significant difference between control and treated group (low and high concentration). The result did not reveal significant difference between high concentration 50 and 500 µg/kg. The sperm count was decreased significantly (p□0.05) in treated group compared to the control in dose dependent manner.

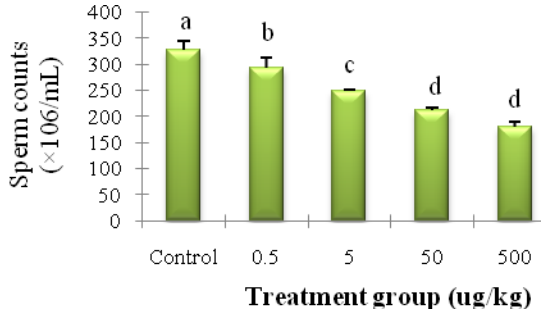

Figure 6. Effect of PCBs on sperm count (×10^6/mL) in the mouse.

Data are represented as mean±S.E. The experiment showed individual significant difference (b, c; P□ 0.05) between low concentration (0.5 and 5µg/kg) and did not reveal significant difference in high concentration (d). The result showed significant difference in treated group (b, c, d; P□ 0,05) in comparison to control group (a). Treatments not sharing a common letter are significantly different (P < 0.05)

Sperm abnormality

The figure 7 and table 3 showed that Sperm abnormality was increased by PCBs treatment group in dose dependent manner. There was a significant increased (P < 0.05) between high concentration groups (500µg /kg and 50g/kg). Also, there were significant difference between control, low concentrations and high concentrations.

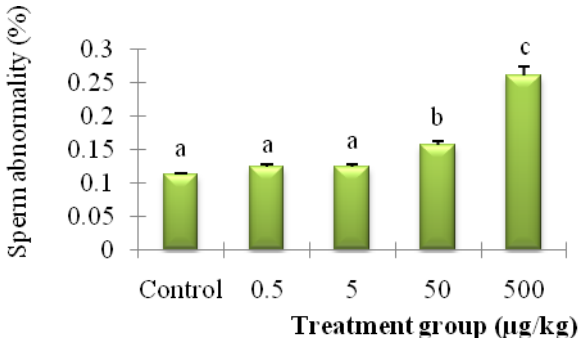

Figure 7. Effect of PCBs on sperm abnormality in control and treated group mice.

Results are expressed as mean ± standard error (SE) of three animals per group. b,c; P□0.05 vs control. Treatments not sharing a common letter are significantly different (P < 0.05)

Table 2: Effect of PCB in testis, epididymis, body weight and sperm of male mice

Treatment group	Control	0.5 µg/kg	5 µg/kg	50 µg/kg	500 µg/kg
Body weight (n=21)	22.69±0.43g	21.26±0.44g	22.37±0.43g	22.74±0.39g	21.64±0.50g
Testis weight (n=21)	0.21±0.003g	0.21±0.004g	0.22±0.004g	0.22±0.005g	0.21±0.006g
Testis/Body Weight (n=21)	0.0093g±0.0001g	0.0099±0.0003g	0.0098±0.0002g	0.0095±0.0002g	0.0095±0.0003g
Epididymis weight (n=21)	0.048±0.001g	0.049±0.002g	0.047±0.001g	0.046±0.001g	0.046±0.001g
epididymis/body weight (n=21)	0.21±0.21%	0.24±0.016%	0.21±0.009%	0.20±0.009%	0.21±0.008
Sperm mortality (*n* = 4)	0.27 ± 0.029^{a}	0.348 ± 0.029^{ab}	0.338 ± 0.030^{ab}	0.360 ± 0.044^{ab}	0.445 ± 0.03^{b}
Sperm count ($\times10^{6}$/mL) (*n* = 4)	326.83 ± 18.30^{a}	294 ± 18.30^{b}	247.42 ± 2.79^{c}	212.33 ± 3.78^{d}	180.83 ± 7.66^{d}
Sperm abnormality (%) (*n* = 4)	0.11 ± 0.003^{a}	0.12 ± 0.003^{a}	0.12 ± 0.005^{a}	0.15 ± 0.004^{b}	0.26 ± 0.01^{c}

Data are expressed as mean±S.E. Treatments not sharing a common letter are significantly different at $p < 0.05$, was assessed by one-way ANOVA followed by Tukey test.

III.2. HISTOPATHOLOGY

Mouse testes (50-day treatment) were studied to evaluate the effect of PCB on histopathology of testicular tissue (spermatogenic cell), which include seminiferous tubule diameter, count the number of germ cell and sertoli cell. In this work were analyzed 5 group of mouse testis (triplicate, 3 in each group) and these groups include the control and testis treated with 0.5; 5; 50; 500 mg/kg/day.

III.2.1. Quantitative Measurement:

This was accomplished by measuring different parameter like:

III.2.1.1. Seminiferous tubule diameter

Table 9 and figure 8, the result showed that there was significant increase ($P < 0.05$) in

seminiferous tubules diameter of Aroclor 1254 treated group 50mg/kg and 500 mg/kg compared to the control group.

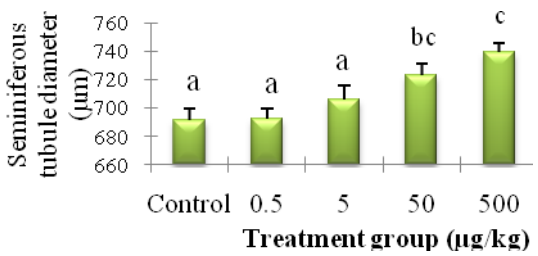

Figure 8. Effect of PCB on seminiferous tubule diameter.

Data is expressed as Mean±SD. Treatments not sharing a common letter are significantly different ($P < 0.05$).

III.2.1.2 - Spermatogenic cell

Spermatogonia

The mean spermatogonia in testis of PCB treated group show non-significant difference compared to control, there was no change of cell between the groups. The number of spermatogonia cells appear decreased in 5, 50, 500 Aroclor treated group but not significant (table 3 and fig 9).

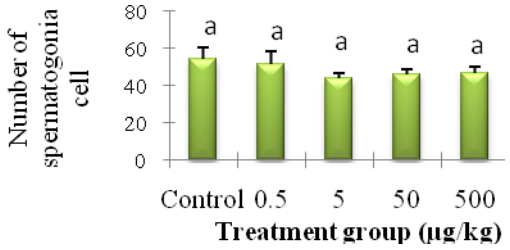

Figure 9. Effect of PCB on Spermatogonia cell.

Data is expressed as Mean±SD. Treatments not sharing a common letter are significantly different ($P < 0.05$)

Spermatocytes

In seminiferous tubules, the number of spermatocytes was counted in control and PCB treated 0.5, 5, 50 and 500 mg/kg group. In table 3 and figure 10, the mean values indicate that there was significant differences between control and high concentration 500 mg/kg.

There was significant decrease (P□ 0.05) in number of spermatocytes of testes in PCB treated group 500 mg/kg as compared to the control and 0.5, 5, 50 mg/kg treatment group.

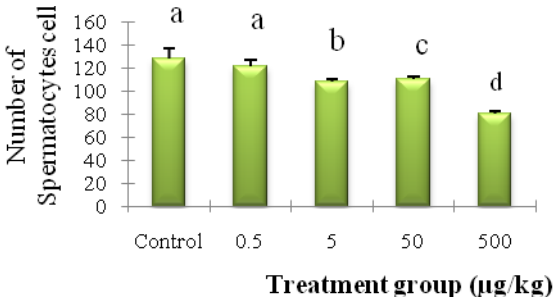

Figure 10. Effect of PCB on Spermatocytes cell.

Data is expressed as Mean±SD. There was significant (P<0.05) decrease of spermatocyte in treated 500 mg/kg group comparison to the control. Treatments not sharing a common letter are significantly different (P < 0.05)

Spermatides

The table 3 and figure 11 showed the mean spermatides of control group was **and** Aroclor 1254 treated group. There was significant decrease (P□ 0.05) in spermatides treated group 50 mg/kg, and 500 mg/kg compared to the control.

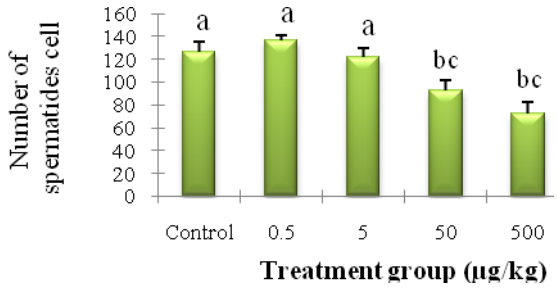

Figure 11. Effect of PCB on Spermatides cell.

Number of spermatides was significantly (P<0.05) decreased in treated groups than the control. Data is expressed as Mean±SD. Treatments not sharing a common letter are significantly different (P < 0.05)

Sertoli cell

The Sertoli cell number of control and PCB treated group are showed in table 3 and fig 12. The number of sertoli cell increased when the concentration of PCB increased in treated group. The mean values of sertoli cell of control group and treated group 0.5

mg/kg and 5 mg/kg indicate that there was no significance (P □ 0.05). It was noted that the mean of sertoli cell in treated group 50 mg/kg and 500 mg/kg were increased significantly (P□ 0.05) in comparison with control group. In figure below we can see that there was no significant difference between high concentration 50 and 500 mg/kg.

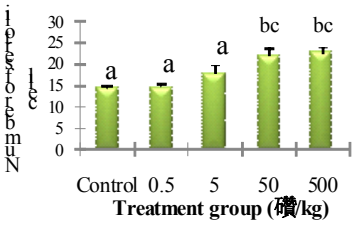

Figure 12. Effect of PCBs on Sertoli cell.

Data is presented as Mean±SD. There was significant increase (P□ 0.05) comparison between treated groups than control. Treatments not sharing a common letter are significantly different (P < 0.05)

Table 3 – Effect of PCBs on number of different types of spermatogenic cell and sertoli cell

Treatment group	Control	0.5 mg/kg	5mg/kg	50mg/kg	500 mg/kg
Diameter seminiferous (mean±SD)	691.04±8.22^{a}	692.36±7.46^{a}	705.65±9.69^{ab}	722.96±8.57^{bc}	739.46±6.20^{c}
Spermatogonia (mean±SD)	54.00±6.51^{a}	51.50±6.58^{a}	44.06±2.92^{a}	45.76±2.75^{a}	46.96±2.90^{a}
Spermatocytes (mean±SD)	128.09±8.77^{a}	121.71±5.36^{ac}	107.90±2.61^{b}	109.63±3.21^{cb}	80.36±2.18^{d}
Spmatides (mean±SD)	126.76±4.69^{a}	136.26±8.56^{a}	121.38±8.77^{a}	93.16±8.58^{bc}	72.66±9.69^{bc}
sertoli cell (mean±SD)	14.33±0.65^{a}	14.60±0.69^{a}	17.73±1.91^{a}	21.97±1.74^{bc}	22.60±1.22^{bc}

Data are expressed as mean±S.E. Treatments not sharing a common letter are significantly different (P < 0.05), was assessed by one-way ANOVA followed by Tukey test.

III.2.2 - Qualitative measurement – Histomorphology

In control and treated groups, different types of qualitative changes were observed.

• **Control***:*

The histomorphological characteristics of testicular tissue studied include the measurement of seminiferous tubules; the number of germ cells and the sertoli cells (Figure 13). In this study, it is indicated that the testes were surrounded by a dense connective tissue layer tunica albuginea. The inner tissue consisted of several round and elongated seminiferous tubules and between theses tubules leydig cells were scattered in the interstitium.

The **Seminiferous tubules** were enclosed by a thick basal lamina and surrounded by few layers of smooth muscle cells. The inside of the tubules are lined with seminiferous epithelium, which consists of two general types of cells: spermatogenic cells and Sertoli cells. These cells were located on a basement membrane. Round or spherical cells were large in size and have darkly stained nucleus, these cell were identified as spermatogonia which are described below.

Spermatogonia were the first cells of spermatogenesis and they were always in contact with basal lamina of the tubule. Two types of spermatogonia can be distinguished in the mouse seminiferous epithelium. Type A spermatogonia had a rounded nucleus with very fine chromatin grain and one or two nucleolus. They were stem cells which divide to form new generations of both type A and Type B spermatogonia. Type B spermatogonia had rounded nuclei with chromatin granules of variable size, which often attach to the nuclear membrane, and one nucleolus. They divide repeatedly, did not function as stem cells and their final mitosis always resulted in the formation of spermatocytes, spermatides and spermatozoa.

Primary spermatocytes: Spermatogonia type B were prolifering by mitosis and resulted in to small size cell designated as Primary spermatocytes. These cells lay in the cell layer luminal to the spermatogonia. They appeared larger than spermatogonia. A large number of primary spermatocytes were always visible in cross-sections through seminiferous tubules. The cells stayed connected by bridges of cytoplasm. The completion of the first meiotic division results in the formation of spermatides. Primary spermatocytes were also dividing originated secondary spermatocytes, located between

primary spermatocytes and spermatids.

Spermatids lay in the luminal part of the seminiferous epithelium. They were small with an initially very light (often eccentric) nucleus. The chromatin condensed during the maturation of the spermatids into spermatozoa, and the nucleus became smaller and stains darker.

Sertoli cell were far less numerous than spermatogenic cells and they possessed long and branched cytoplasmic projections towards the lumen of the seminiferous tubules. They were present on the basement membrane and in between the germ cells or dividing spermatogonia. They had characteristic irregular nucleus and its position was not fixed in the cytoplasm. The chromatin material was evenly distributed and their cytoplasm was granular.

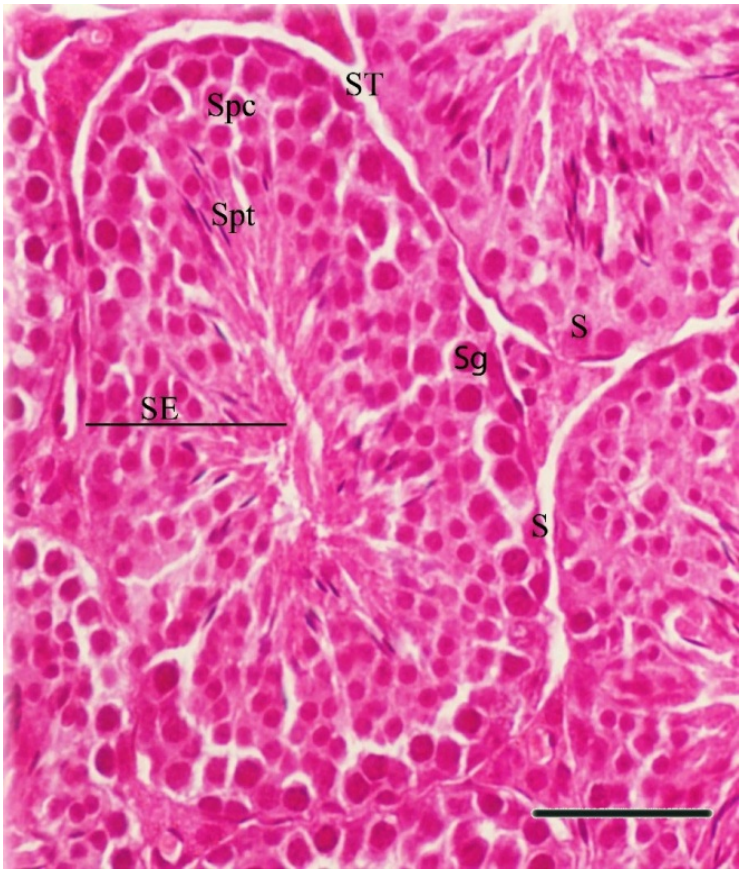

Figure 13. **Photomicrograph of seminiferous tubules of control mouse.**

The seminiferous epithelium (SE) containing spermatogonia, spermatocytes (Spc) Spermatides and sertoli cell (S). Note the defoliating of cells from germinal epithelium. H&E staining. Scale bars: 50µm.

• Testis treated with low dose of PCBs (0.5 and 5 mg/kg)

The treated animal with 0.5 and 5 mg/kg of PCB did not reveal significant alteration compared with the control (Figure 14 and 15).

Seminiferous Tubule: Interstitial tissues were compact as seen in control testis. Each tubule was surrounded by peritubular cells which had elongated nuclei. Seminiferous tubule diameter increased but not significantly compared to the control. The organization of spermatogenic cell and sertoli cell in PCB treated group were the same comparison with control group. The germ cells and sertoli cells were located on the basement membrane.

Spermatogonia: Seminiferous epithelium was lined by basal lamina and it was composed of spermatogonia and sertoli cells. Spermatogonia type A was identified as large and oval cells with darkly stained nucleus attachement to the basement membrane. Their cytoplasm was granular. Spermatogonia type B were round and spherical in shape.

Spermatocyte: Division of spermatogonia type B by mitosis has produzed primary spermatocites as observed in the control group. They were located close to the lumen of seminiferous tubules, and their spherical nuclei contain a homogenous chromatin.

Spermatides: Spermatids were located close to the luminal aspect of the seminiferous epithelium. These cells were round or spherical and smaller in size than secondary spermatocytes. In addition to round spermatid, elongated spermatids with small tails were observed which were very conspicuous and had initiated spermiogenesis.

Sertoli cell: In low dose (0.5 and 5 mg/kg) the sertoli cells were also distributed between the developing spermatogenic cells present in seminferous tubule similar to control seminifeous tubule. These cells contained long and branching cytoplasmic that included an irregular and proeminent nucleus, which extends to the lumen. Chromatin was granular. Cytoplasm was light in color.

• Testis treated with high dose of PCBs (50 and 500 mg/kg)

Seminiferous tubule: The testes treated with high concentration (50 and 500 mg/kg) showed seminiferous tubules with significant degenerative alterations, such as: significant exaggerate intercellular space, reduction of spermatogenic cell, a lot of nuclei appeared pycnotic. Tubule diameter in high dose were (722.96±8.57), (739.46±6.20) and these mean were significantly different from control and low concentration 0.5 and 5

mg/kg. Diameter of seminiferous tubule increase significantly (P 0.05) in high concentration compared to the control group.

Spermatogonia: The spermatogonia in high concentration were embedded in the basement membrane as in control and 0.5 and 5 mg/kg treatment group seminiferous tubule.

Spermatocytes: Mitotic division in spermatogonia type B has produced daughter cells designated as primary spermatocytes as observed in the control epithelium. These cells were characterized by a spherical nucleus and they have round nucleus. In high concentration were observed significant decreased (P 0.05) of spermatocytes cell comparison than control group.

Spermatides: spermatids in high dose PCBs testis were markedly decreased, when compared to the control.

Sertoli cell: The sertoli cells in treated group (50mg/kg and 500 mg/kg) were present in seminiferous epitethelium between the spermatogenic cell similar to control and low concentration (0.5 and 5µg/kg). These cells possessed long and branched cytoplasmic projections which extend to the lumen. An irregular and proeminent nucleus was present in the cytoplasm and the chromatin was granular. Cytoplasm was light in color. In treated group high concentration, these cells increased significantly (P 0.05) comparison than the control and low concentration.

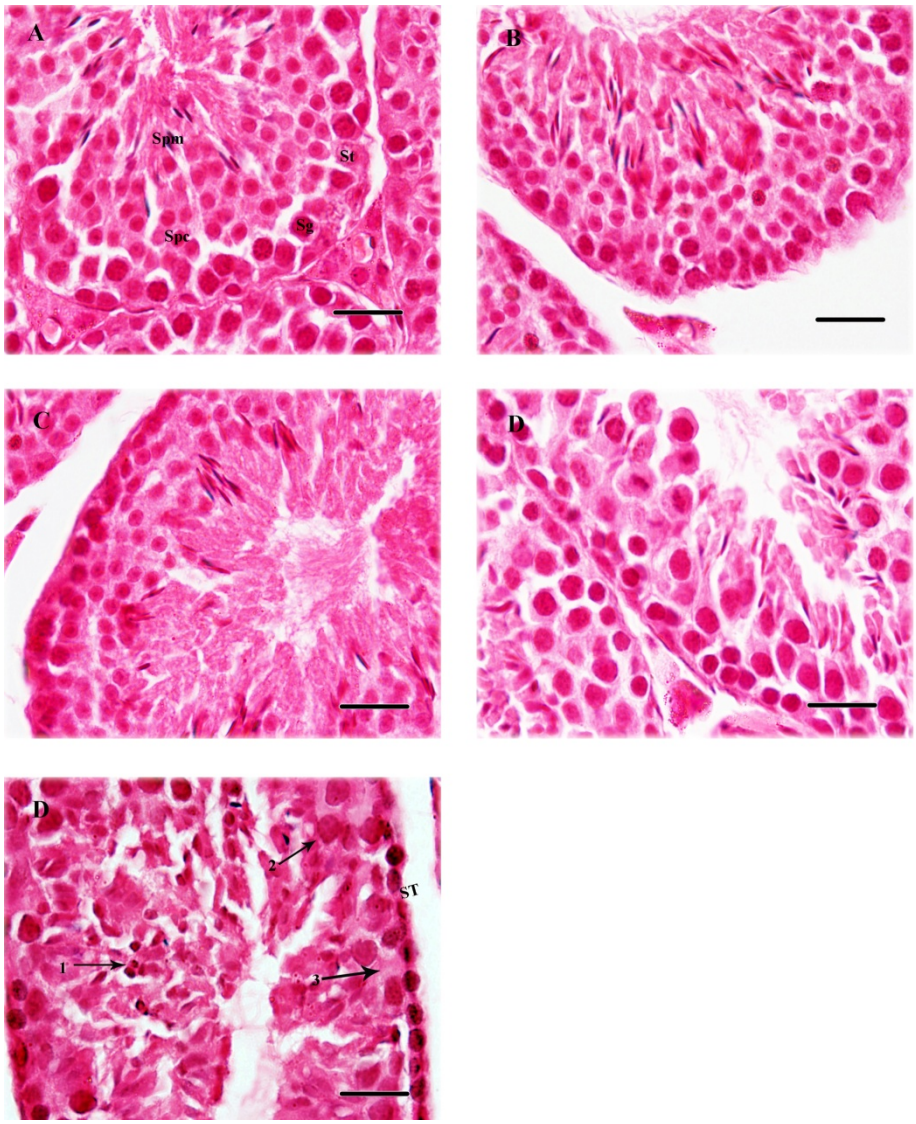

Figure 14. Comparison of seminiferous tubules from mice treated with PCBs.

The photomicrograph showed a part of seminiferous tubule (ST) from control mouse (A), and B and C from **0.5, 5** mg/kg. In D (50 mg/kg) and E (500 mg/kg) the seminiferous tubules showed significant alteration in seminiferous epithelium. Although in E, a lot of nuclei appear picnotic (1), significant exaggerated intracellular space (3), reduction of spermatogenic cell and few spermatides(2). Note the defoliating of cells from germinal epithelium. H&E staining. Scale bars: 20 µm.

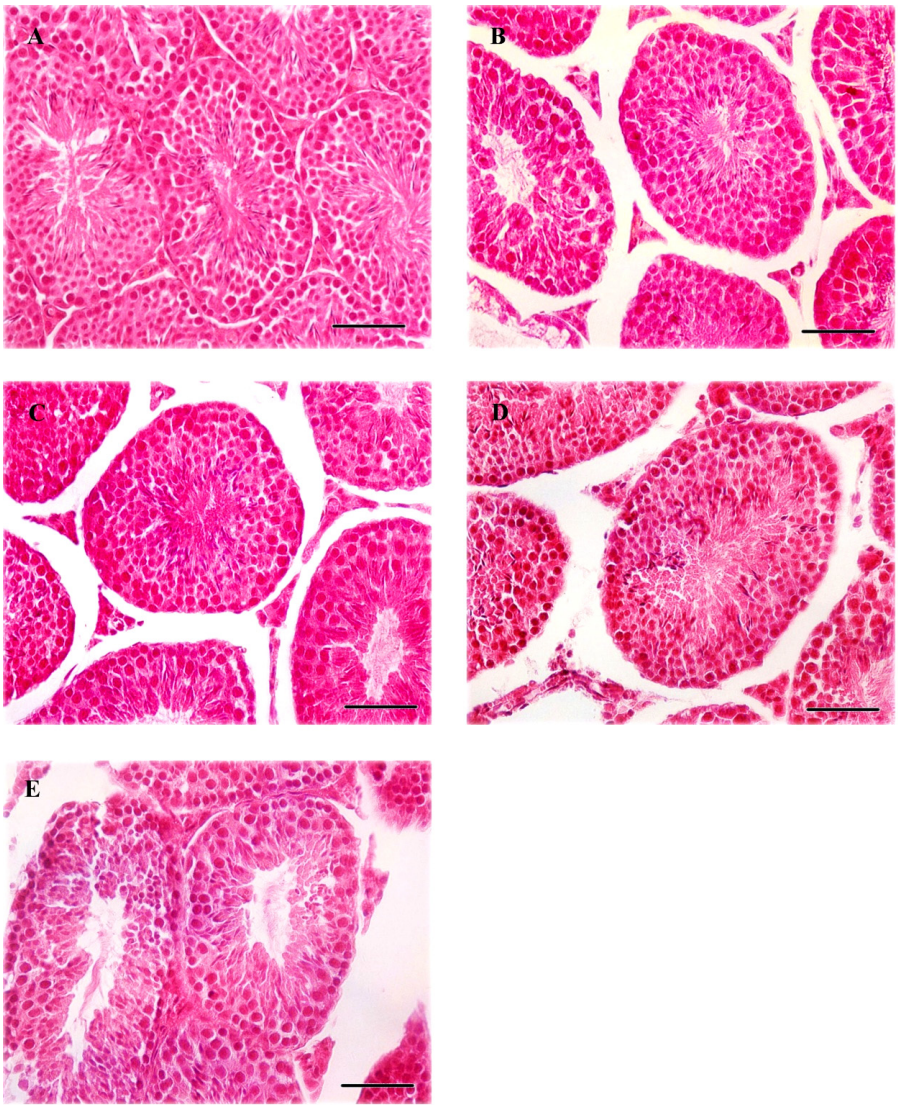

Figure 15. Histological effects of Polychlorinated biphenyls (PCBs) on mice.

(A) Seminiferous tubules from a control showing normal spermatogenesis. (B, C, D and E) Seminiferous tubules from 0.5, 5, 50 and 500 µg/kg PCB-treated mice respectively. In high concentration 50 µg/kg (D) and 500 µg/kg (E) the seminiferous epithelium are disrupted and there are a few sperm heads. Note the defoliating of cells from germinal epithelium. H&E staining. Scale bars: 50 µm.

III.3. WESTERN BLOT

The level of protein on testis was measured using western blot analyses. Five groups of individual mouse testis (3 testes for each group) were analyzed. These include the control and testes treated groups (0.5, 5, 50 and 500 µg/kg). Testes of five groups of individual mice were homogenized separately, (3 testes in each group). The supernatant obtained was used for further study.

III.3.1. Determination of protein quantification

From de BSA standard dilution was obtained a standard curve to determine the amount of protein in each sample. An amount of 0.8 µl sample was used in each dilution to prepare loading sample.

III.3.2. Effect of PCBs on the PCNA expression in testes

The level of PCNA in control group was 0.57± 0.21 folds; although in treated group it were 0.73±0.14, 0.82±0.18, 0.89±0.05, 0.71±0.09 folds of optical density of target protein. The testes of mouse have the normal complement of germ and somatic cells. Western blot analyses of control and treated mice testis were examined for the presence of PCNA proteins with Antibody to PCNA. The results of this experiment clearly show that the antibody detected the presence of PCNA, but there was no significant difference between control and treatment group (fig 16& 17).

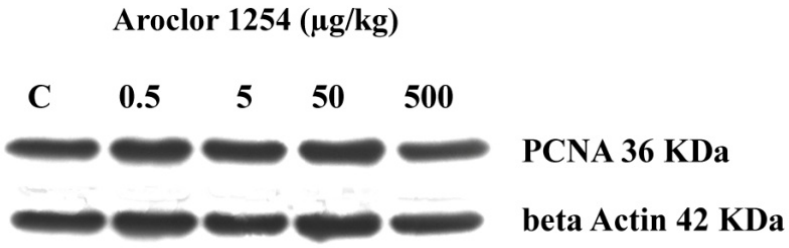

Figure 16 - Western blotting analysis of PCNA in the testes of male mice treated by PCBs.

A Representative chemiluminescent detection of PCNA expression was shown.

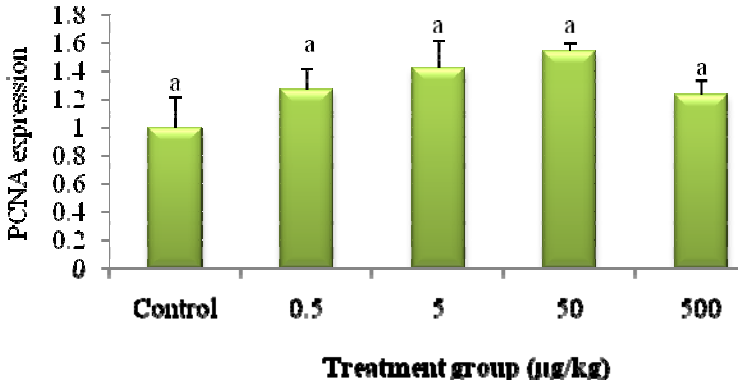

Figure 17. Analysis of PCNA expression by Western blot analysis in the testes of male mice treated with Polychlorinated biphenyls (PCBs).

The intensities of PCNA protein bands were quantified by densitometry, respectively. Results were expressed as folds of optical density of target protein and the β-actin determined in control. The mean protein expression from control was designated as 1 in the graph. Values (means±S.E.) are representative of data obtained in three independent experiments (n=3). The result showed that there was not significant different at $P < 0.05$ as assessed by one-way ANOVA followed by the duncan test.

III.3.3. Effect of PCBs on the expression of ER-α, ER- β, AR in testicle

Photomicrographs of ER-α, ER- β, AR with a representative control and PCBs treated group of testis mice are shown in the figure (figure 17). The testicular ER alpha in control group was 0.997953±0.088666 folds and in PCBs treated group were 0.93±0.20, 1.16±0.26, 1.09±0.34, 1.18±0.30 folds of optical density respectively. There was no significant difference in level of ER alpha between control and treated group in mice testis. The level ER beta protein in control group was 0.84±0.24; although in treated groups were 0.69±0.22, 0.86±0.21, 0.84±0.19, 0.68±0.35 folds of optical density.

The result showed that there was no significant difference between control and treated group. In relation of AR to the level of hormone in control group, it was 0.78±0.46; however in treated group it was 0.83± 0.50, 0.73± 0.44, 0.90± 0.55, and 0.93± 0.47. There was no significant difference between the control and treated groups.

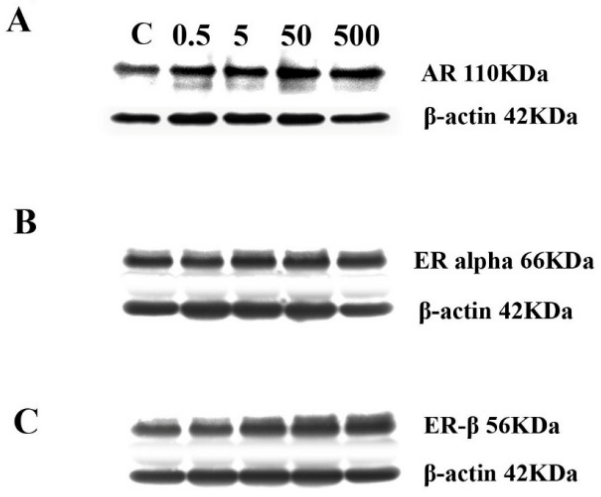

Figure 18. Effect of Polychlorinated biphenyls (PCBs) on expression of ER-α, ER-β, AR in testes of male mice as estimated by Western blotting.

In this picture was shown a representative chemiluminescent detection of ER-α, ER-β, AR expression. Treatments that did not share a common letter are significantly different at $P < 0.05$ as assessed by one-way ANOVA followed by the duncan test.

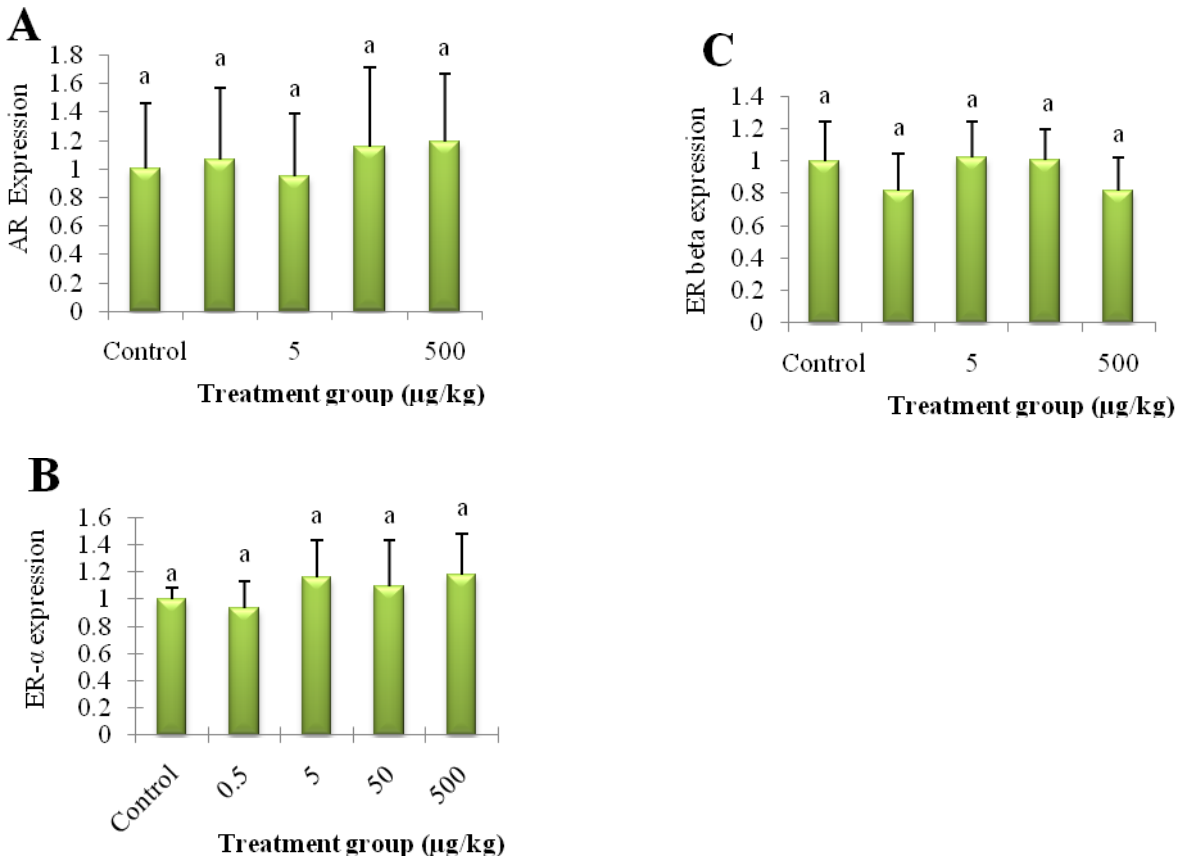

Figure 19. Quantification of Intensities of ER-α, ER-β, AR target protein bands by densitometry.

Intensities of target protein bands (figure A, B and C) were quantified by densitometry, respectively. Results were expressed as folds of optical density of target protein and the β-actin determined in control. The mean protein expression from control was designated as 1 in the graph. Values (means±S.E.) are representative of data obtained in three independent experiments (n=3). Treatments that did not share a common letter are significantly different at $P < 0.05$ as assessed by one-way ANOVA followed by the duncan test.

III.4. EFFECT OF AROCLOR 1254 ON THE LEVEL OF TESTOSTERONE AND 17B-ESTRADIOL OF TESTIS IN MALE MICE

The result showed that the mean concentration of testosterone in control and Aroclor 1254 treated group (0.5, 5, 50 and 500 µg/kg) were 39.06±10.99, 20.10±1.42, 24.77±6.37, 38.55±8.56, 43.67±2.56 ng/g; however the mean concentration of 17β-estradiol in control group was 59±9.29 pg/g and treated group were 62.66±12.81; 40±18.67; 58.25±15.33, 49.5±7.27 pg/g respectively.

The result demonstrated that Aroclor 1254 did not cause significant alteration of testosterone level in testes compared to the control. In contradiction, Aroclor 1254 treated group result in a significant increase of testosterone level in low concentration (0.5 µg/kg) compared to the high concentration (500 µg/kg) (Figure 19). The 17β-estradiol level did not show any significant difference in Aroclor 1254 treated group in dose dependent manner compared to the control (figure 20).

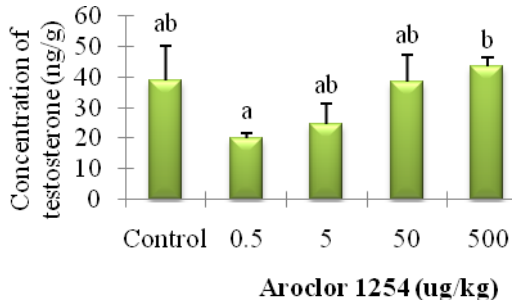

Figure 20 – Level of Testosterone in testis treated with Aroclor 1254. (n=4).
Treatments not sharing a common letter are significantly different (P < 0.05)

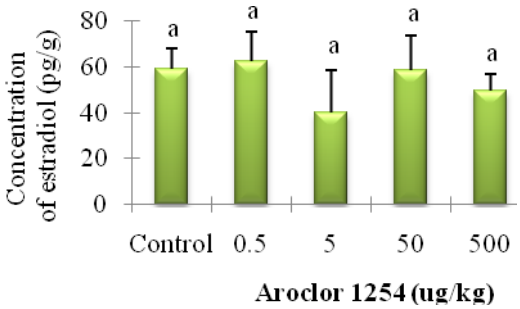

Figure 21 - Level of 17β-estradiol in testis treated with Aroclor 1254. (n=4).

Treatments not sharing a common letter are significantly different (P < 0.05)

CHAPTER IV- DISCUSSION

In recent years, it is observed that humans are exposed to various chemicals [69]. PCBs, one of these chemical, are present in human milk worldwide with considerable concentration specifically in industrialized countries, and the developing breast-fed infant belongs to a special risk group exposed to these toxic substances[2]. Majdic (1998) cites, in recent years, several reports about the increase in male reproductive problems like cryptorhidism, hypospadias and testicular cancer in western countries. Also, several reports have shown a decline in average sperm counts, which might be as high as 50% in western males in the last 50 years. Although the data about lower sperm counts are still highly debated and will be difficult to prove, the data about increased incidence of cryptorhidism, hypospadias and testicular cancer is most likely to be real [51].

IV.1. EFFECT OF AROCLOR 1254 ON MORPHOLOGICAL PARAMETERS OF MALE MOUSE

Body weight:

Sander et al (1977) show that PCB feeding did not affect final body weights or the weights of the reproductive organs [16, 70]. This was also observed in other studies where adult rats were treated with PCB153. In contrast, body weight appears to be a more sensitive indicator of toxicity when PCB treatment occurred during prenatal and early postnatal stages [71]. In rats and adult cocks PCB had no significant effects on body weight and some physiological parameters including heart rate, respiration rate, body temperature and the number of red and white blood cells over a period of six weeks; but the reproductive function was severely damaged. These results indicated that the gonad was a sensitive target for disruption by PCB [72]. In contrast, low dose exposure of PCB in the reproductive system of male goats exposed during the gestation and lactation periods exhibit a significant higher body weight in treated animal compared with the control group [30].

Olsson et.al (1998) reported that birth weights of rhesus monkeys (*Macaca mulatta*) decreased in offspring receiving approximately 0.04 mg Aroclor 1016/kg b.w./day in a

chronic study. But the use of this study for regulatory purposes has been questioned [73]. In the present study, the mice fed with Araclor 1254 at low concentration (0.5 and 5 µg/kg) and at high concentration (50 and 500 µg/kg) did not reveal significant deference in body weight compared to control group.

Testis weight, epididymis weight, sperm count, mortality, abnormality:

In a recently published study, Murugesan et.al (2007) demonstrated that exposure to PCBs may cause several defects in spermatogenesis as well as reduced weight of testis and accessory sex organs in adult rats. In rhesus monkeys, exposure to PCBs resulted in reduced sperm counts and decreased cellular contents in the seminiferous tubules. In human, the levels of PCBs have been inversely correlated to the sperm number and motility [74].

Previous studies showed that male Holtzman rats exposed to Aroclor 1254 through early lactation, from birth up to day 9, demonstrated decreased fertility at 18 weeks of age and increased testis weight at 23 weeks of age. This decline in fertility was not accompanied by a decrease in epididymal sperm count or changes in sperm morphology or motility, but a decline in the ability of sperm to fertilize eggs. Neonatal exposure of Sprague-Dawley rats to Aroclor 1254 and 1242, from birth up to day 25, increased testis weight and daily sperm production at 19 weeks of age. In contrast, Aroclor 1242 can cause change in spermatogenesis and reproductive development in males exposed during gestation and lactation [16]. Recent studies have documented that PCB reduced adult testis size and sperm production following prenatal and neonatal exposure to estrogenic chemicals [16].

Gestational and lactational exposure of 16-week old mice to Aroclor 1242 resulted into increased average of sperm count; even the magnitude of the effect was smaller than that observed in rats. In contrast to the effects in rats at 16 weeks of age, testis weight was not significantly increased [16, 75]. However, in uterus exposure of Dunkin Hartley guinea pigs to 2-3 mg clophen (A50/kg) maternal body weight/day between GDs 18 and 60 decreased both in absolute and relative testis weights[75]. Neonatal treatment with Aroclor 1242 or 1254 significantly increased adult rat testis size

and sperm production. This information is the first to report that environmental contaminants such as PCBs can produce these types of testicular changes [32].

Oral exposure of Aroclor 1254 in female rats on days 1, 3, 5, 7, and 9 after birth showed that the sperm of their male offspring reduced in their capacity to fertilize eggs. There was no alteration in production, morphology or motility of epididymal sperm, in association with the reduced ability to fertilize eggs. Daily neonatal exposure of Aroclor 1242 or 1254 from day 1 to day 25 in rats resulted in increased adult testis weight and sperm production. Aroclor 1254 was more potent than 1242, increasing testis weight of 13 and 23 % and in daily sperm production of 27 and 42 % at doses of 0.4 and 1.6 mg/day [73].

The effect of PCB 1254 on rat sperm production was observed in previous studies. This effect induces hypothyroidism in neonatal rat and has also been shown to increase adult testis weight and daily sperm production. However, acute treatment of some noncoplanar congeners and Aroclor 1242 can decrease serum thyroxine concentration. One hypothesis is that the PCBs and their hydroxylated metabolites compete with thyroxine for binding to transthyretin, which results in an increase in the free fraction of thyroxine, thus increasing conjugation and excretion [16].

In this study, the result demonstrated that male mice fed with Aroclor 1254 did not induce any alteration in testicular and epididymis weight. However, the sperm count of Aroclor 1254 treated group has shown significant decrease compared to control. Also, the result showed a significant increase of sperm mortality at 500 µg/mg and sperm abnormality at high concentration during maturation period in male mice when fed for 50 days.

IV.2. EFFECT of AROCLOR 1254 on HISTOPATHOLOGICAL PARAMETERS of TESTIS

Sertoli cells

Olsson et al. have shown in their work that male rat exposed to Aroclor 1242 or 1254 (from day 1 to day 25) induce hypothyroidism, which leads to increased Sertoli cell proliferation, testis weight, and daily sperm production [73]. These increases in testis

weight were most likely due not to AhR agonist in the Aroclors but to other PCB congeners which alter thyroid hormone status, as transient hypothyroidism early in postnatal development has been shown to increase Sertoli cell number, sperm production and testis size [75]

Cook et.al (1996) report that PCB treatment is efficient in increasing adult testis size and DSP if treatment is started at birth, but not if it begins at 12 days of age [32]. A lot of research shows that the effects of PCBs on these parameters are modest and can vary depending on the mixture, dosage, and time of exposure. [76]

Previous studies showed that the number of sertoli cells was not reduced in all PCB-treated groups and the functional disturbance of sertoli cell was unclear. Further detailed investigations concerning the interactions between Sertoli cells and spermatides following PCBs exposure are required [77]**.** A lot of evidence supports the hypothesis that PCB effects on testis weight and sperm production result from hypothyroidism produced by these chemicals [32].

Previous studies indicated that PCB-77, PCB-22, octylphenol and floranthene were directly toxic to isolated neonatal Sertoli cells in a dose dependent manner for 24 h in vitro. Sertoli cells, which form the somatic component of the seminiferous tubules, are a major determinant of sperm production by the testis. The result showed that the percentages of viable cells were significantly reduced in a dose and time dependent manner, and this reduction in Sertoli cell number leads to subnormal supply of sperm [3] . In this work the number of sertoli cells on seminiferous epithelium increased significantly in treated group male mouse comparison than control.

Seminiferous tubules

The seminiferous tubules suffered significant degenerative alterations in the testes of animals exposed to Aroclor 1254 [27]. Wakui et.al (2007) report that the number of Stage VII-VIII seminiferous tubules in rats prenatally exposed to PCB126 was significantly increased at 7 and 17 weeks of age in the 7.5 μg exposure group [77]. In this research seminiferous tubule diameter in treated mouse increased significantly at high concentration (50 and 500 μg/mg) when compared to the control. The effect of Aroclor was also studied on histomorphology of seminiferous epithelial cell of mouse. Large

intertubular spaces were observed between the seminiferous tubules in Aroclor 1254 treated testes at high (50 and 500 µg/mg) concentration compared to the control.

Spermatogenic cells

Wakui et.al (2007) demonstrated that rat group treated with 7.5 µg PCB at 7 and 17 weeks of age showed significant decrease in the numbers of spermatids at all developmental stages and decrease in meiosis without significant difference. In this stage occurred inhibition of the development of spermatocytes to spermatids. They explained that a significant alteration in sperm number in the cauda epididymis was not observed in any PCB-treated group. The PCB effect on the number of spermatids has been suggested to indicate interference with spermatogenic process, while an effect on sperm number in the cauda epididymis may not be specific to an alteration of the spermatogenesis process[77].

The present study demonstrated that Aroclor 1254 causes significant alterations of spermatogenic cell. There was no significant alteration in the number of spermatogonial cells of treated mice group compared to control. In contrast, the number of spermatocytes (500µg/mg) and spermatidis (50 and 500 µg/mg) in treated group decreased significantly in comparison to control.

IV.3. EFFECT OF AROCLOR 1254 ON PCNA AND STEROID HORMONES (AR, ER ALPHA/BETA) IN TESTIS

PCB1254 did not alter the level of PCNA in mouse testis

The PCNA is essential for multiple cell-cycle pathways, as the initiation of DNA duplication. This protein is a useful molecular marker for assessing germ cell kinetics, and the staining intensity could evaluate the testes spermotogenic function of male infertility, including proliferation of the cells [66]. PCNA was identified in the mitotically proliferating spermatogonia, but not in spermatocytes which had just entered meiosis[78]. In the present work Aroclor 1254 did not alter the level of PCNA expression on spermatogonia cell in testis of mice.

PCB1254 did not alter the level of ER alpha, ER beta and AR in mouse testis

In various cell types of the mammalian testes have been discovered two types of estrogen receptors (ER α and ER β). The ER showed distinct patterns of cellular expression with a prevalent distribution of ER α in Leydig cells; and ER β in Sertoli and germ cells of most mammals[61]. The PCB have been shown to mimic estrogenic affects with an active competition for estrogen receptors and may cause similar modifications to those resulting from an overproduction of estrogen[79].

Chen et al. (2008) report that the effects of estrogen and androgen on target tissues are mediated by ER and AR, which are ligand-activated transcription factors that regulate the expression of target genes. On the reproductive system the AR and ER may be an important part of the mechanism of testicular development, and the transgenic mice with deficient estrogen receptors were infertile due to disruptions to spermatogenesis, reduced epididymal sperm content, reduced sperm motility and elevated fluid production in the efferent ducts of the testes [66]

PCB can bind to one of several steroid receptors (glucocorticoid, androgen, estrogen, etc.), but the PCB complex may fail to elicit a response. This response to this hormone is characterized by the synthesis of proteins essential to spermatogenesis. Then, if the response were inhibited, spermatogenesis equally would be inhibited, and were observed an effect which could be responsible for the histological changes. These histological alterations certainly are responsible for the depressed fertility observed in Peromyscus resulting from chronic exposure to PCBs [27]

PCBs are known as xenoestrogens. They have estrogenic activity and toxic effect on many physiological functions. In testes of vertebrate, the estrogens are involved in Leydig cell development and regulation of spermatogenic progression. Male mice with deficient functional estrogen receptors exhibit a wide variety of reproductive problems such as infertility, abnormal spermatogenesis, reduced testis size, and decreased sperm motility. On the other hand, estrogens also have negative effects on testicular functions by decreasing androgen production and inhibiting or damaging spermatogenesis. PCBs caused adverse effects on reproductive system and may origin disruption of

spermatogenesis. However, in almost of spermatogenic cells the hyperchromatism nucleus have appeared as a result of treatment by PCBs, it may be deduced that PCBs caused these adverse effects by both estrogenic and toxic actions [72].

The Androgens control male sex behavior and development. After determination of male sex, androgens growth and development of the male reproductive system, including the penis, testes, prostate, sperm, and other essential features [22].

In this study, the administration of Aroclor 1254 did not change the level of testicular AR, ER α/β expression, suggesting that the change of ER may occur on mRNA expression of ER and AR. Campi (2007) report that changes in mRNA levels are not necessarily translated to protein expression. Thus, they examined therefore the estrogen receptors α and β protein levels on human MCF-7 cells by western blot at the same concentrations used for RT-PCR, and their results showed a decreased ERα/ERβ ratio after phosalone treatment [62].

Previous study shows that PCB disrupts the mRNA expression of the hormonal receptors such as AR and ER in Sertoli cells, LH receptor in Leydig cells, epididymis and prostate. The diminished testosterone levels may be a decrease in spermatogenesis and androgen-dependent epididymal sperm maturation. The dysfunction of Epididymal may have a detrimental effect on various cytostructural modifications and biochemical changes during sperm maturation process, which could result in decreased sperm count and motility [80].

Others investigators have demonstrated that alteration in expression genes involved in spermatogenesis can be targeted during developmental exposure to PCBs. This alteration in testicular or epididymal gene expression can decrease sperm fertilizing ability without apparent morphological and motion abnormalities. The cDNA arrays technique may identify PCB-induced alterations in unknown target genes that are associated with changes in sperm functionality, and identification of these genes may lead to biomarkers that predict PCBs induced infertility [81].

IV.4. EFFECT OF AROCLOR 1254 ON TESTOSTERONE AND 17B-ESTRADIOL IN TESTIS OF MALE MICE

Testosterone hormone is synthesized by Leydig cells [82] and is the principal androgen involved in the regulation of normal spermatogenesis in the rat because of its high intratesticular concentration [29]. Robertson et al. (2001) in their previous work have shown that decreased aromatase activity in the hypothalamus of PCB-exposed newborn male rats cause reduced serum testosterone concentrations and testes weights in adult male littermates in addition to feminized sweet preference behavior [81]. However, willingham et al. (2000) report that Aroclor 1242 exposure results in an increase in aromatase activity in the AKG adrenal-kidney-gonad (AKG) of red-eared slider turtle before hatch. After hatch, the levels of testosterone in Aroclor 1242-exposed males are low, and an estradiol level is measurable only in the Aroclor-2142-exposed male hatchlings. For the reason that aromatase is the enzyme that converts testosterone to estradiol, lower testosterone levels and higher estradiol levels are expected outcomes of increased aromatase activity [83]

Yamamoto et al. demonstrated in their study, PCB169 has a stronger inhibitory effect on testicular function than PCB126 in pregnant and lactacional rats. At 3 weeks after birth, the PCB126 reduced the testosterone concentration principally through the inhibitory effect on the higher central nervous system; but exerted little or no inhibitory effects at 6 weeks after birth. In contrast, PCB169 reduced the testosterone concentration at 3 and 6 weeks after birth, indicating a stronger inhibitory effect on spermatogenesis, via the inhibition of the pituitary-testicular axis; but, at 15 weeks after birth, the mRNA for testicular steroid-synthesizing enzymes had undergone changes, resulting in increased testosterone levels. These two kinds of PCBs reduced the percentage of testicular Leydig cells at 3 weeks after birth, indicating the inhibition of Leydig cell differentiation [29] .

Data from previous study suggest that exposure to high levels of PCBs could affect 17β-Estradiol blood levels, although this was not significant. Recent work have demonstrated that a weak but significant negative correlation was found between serum levels of the prevalent PCB 153 congener and testosterone in young men, and 17β-Estradiol concentrations (within a concentration range of 43–144 pM) were also a slight

reduced in the more exposed subjects. A lot of study in rats exposed to PCB mixtures also reported lower testosterone and 17β-Estradiol serum levels and suppression of brain aromatase activity. Decreased 17β-Estradiol concentrations could be associated with AhR activation by dioxin-like PCBs, leading to enhanced CYP1A/CYP1B1-catalyzed metabolism of 17β-Estradiol [84]. In the present study, the testosterone and 17β-estradiol levels in the testes treated with aroclor 1254 were not significantly altered comparison than control group in a dose dependent manner.

CONCLUSION

The present study was to characterize biological effects of Aroclor 1254 on reproductive system of male mouse. The results of this work can be summarized as follows:

The body, testis and epididymis weight did not reveal morphological alteration in PCB 1254 treated group. However, the result showed that the sperm mortality and percentage of sperm abnormality increase significantly and in contradiction have observed a reduction of sperm count (p□0.05) in Male C57 mice.

The histological examination of testis was taken. The result showed significant increase in diameter of seminiferous tubules and sertoli cells. A significant reduction of spermatogenic cell was observed in seminiferous epithelium. The spermatogonia cells appear decreased but not significantly; and the spermatocytes and spermatides cells decrease significantly. These observations have shown that changes in reproductive system affect fertility of mammals and human being.

The PCB (Aroclor 1254) did not cause alteration on level of protein (PCNA) and steroid hormone (ER alpha/beta and AR) on the spermatogenese of male mice. However, in further study the effect of this toxic substance on RNA expression will be investigated. Aroclor 1254 disturb endocrine function, interacting with hormone systems via a number of different mechanisms.

Exposure of Aroclor 1254 did not affect significantly the level of testosterone and estradiol in the testis, but further study on the mechanism and development of male reproductive system is required.

In conclusion, adverse effect of Aroclor 1254 and their mechanism on spermatogenesis will continue to be investigated, and the histopatological studies (Sperm quality and histopathology) of the present study showed that Aroclor 1254 at low concentration treatment has a stronger inhibitory effect on spermatogenesis in testis of mice.

FURTHER STUDY

Further studies are required to define and clarify the detail mechanism of Aroclor 1254 on the testicular development in mice. As a continuation of our current studies, we intend realize the following research in the near future. Our researches will be focalizing in the following step below:

Evaluated the testis and epididymis as a pool because they are two clear target of estrogenic regulation

Analyze hypothyroidism affect the reproductive system of adult male mouse by evaluating testicular morphology and determine the level of ER alpha/beta and AR and thyroxine on thyroid gland after exposure of PCBs

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